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STUDIES ON THE DETERMINATION AND EXCRETION OF ADRENALINE AND NORADRENALINE IN THE URINE

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By
ELJA PITKÄNEN

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STUDIES ON THE DETERMINATION AND EXCRETION OF ADRENALINE AND NORADRENALINE IN THE URINE

BY
ELJA PITKÄNEN

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CONTENTS

Page

PREFACE 5
INTRODUCTION 7
DETERMINATION OF ADRENALINE AND NORADRENALINE IN THE URINE 9
Earlier Investigations 9
Chemical Methods 9
Biological Methods 13
Own Investigations 15
1. Inhibition of the Fluorescence Reaction 17
2. Precipitation in the Eluate 19
3. Specificity of the Method 19
4. Differentiation of Adrenaline and Noradrenaline 27
5. Other Modifications 31
6. Optimal Conditions for the Fluorescence reaction 32
7. The Method 37
8. Accuracy and Recovery. Normal Excretion 39
9. Biological Comparison 40
Discussion 42
STUDIES ON THE INSULIN-INDUCED URINARY OUTPUT OF ADRENALINE IN RATS 47
Earlier Investigations 47
Material and Methods 48
Results 51
1. Adrenaline Excretion and Course of Hypoglycaemia 51
2. Effect of Various Doses of Insulin 53
3. Effect of Hydration 56
4. Effect of Fasting 59
5. Effect of Diet 61
6. Continued Administration of Insulin 64
7. Restoration of Adrenaline Excretion 66
8. Insulin Administered Repeatedly at Short Intervals 68
9. Effect of Adrenalectomy 71
Discussion 74
DETERMINATION OF ADRENALINE AND NORADRENALINE IN THE URINE IN THE DIAGNOSIS OF PHEOCHROMOCYTOMA 82
Earlier Investigations 82
Own Investigations 84
Conclusions 87
STATISTICAL TREATMENT OF CERTAIN DATA 89
GENERAL SUMMARY 92
REFERENCES 94

PREFACE

This work has been carried out at the Department of Medical Chemistry, University of Helsinki. I wish to express my deep and respectful gratitude to my honoured teacher, Professor P. E. SIMOLA, M.D, Ph.D., the chief of the Department, for his continuous valuable advice, encouragement and unfailing support extending throughout this work, and for his kindness in making the Department's working facilities available to me.

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I was permitted to review numerous case histories in several hospitals in Finland, for which I am greatly indebted to the Heads of these hospitals.

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Helsinki, July 1956

ELJA PITKANEN

INTRODUCTION

During the present century adrenaline and noradrenaline, the two main biological representatives of catechol amines, have been extensively studied and today a great deal is already known concerning their occurrence and significance in the animal organism.

Adrenaline, isolated in 1901 by TAKAMINE, is the principal hormone of the human suprarenal medulla. Its secretion is low during normal activity but a discharge occurs under conditions where the metabolic needs of the tissues are increased, as in hypoglycaemia, hypoxia and in emergency states. Besides its effects on the blood circulation, the most outstanding characteristic is its hyperglycaemic action. It is postulated that this is caused by a mobilisation of the liver glycogen stores due to increased phosphorylase activity (SUTHERLAND and CORI 1951). Adrenaline is considered to play an important rôle in the homeostatic mechanism of the regulation of the blood sugar level.

The importance of noradrenaline for the animal organism was discovered much later, principally through the studies of U. S. v. EULER in the 1940's, although this substance was synthesised nearly fifty years ago. Noradrenaline has been shown to be the chemical transmitter of nerve impulses in the sympathetic nervous system and it is thus intimately connected with the physiological and chemical regulation of the blood pressure and circulation.

Side by side with the investigations on the chemical and biological properties of these substances, attempts have been made to work out methods for their determination in the blood and the urine. The most extensive studies have been made with blood. As the specificity of the methods increased progressively it was observed that the adrenaline and noradrenaline content of especially peripheral blood is too low for accurate measurement (PEKKARINEN 1948, LUND 1950). The research work

with urine, on the other hand, is of more recent date and has been more successful. Adrenaline and noradrenaline, like many other hormones, are present in the urine in far greater concentrations than in the blood. It has therefore been possible to determine the variations in the urinary adrenaline and noradrenaline excretion even within the physiological range.

Conclusive evidence for the occurrence of adrenaline in the urine was presented by RICHTER (1940), who after oral administration found this substance to be excreted in considerable amounts. HOLTZ *et al.* (1947) showed that biologically active catechol amines occur also in normal urine, partly in a conjugated form. A biological method for the determination of adrenaline and noradrenaline in urine was introduced by v. EULER and HELLNER (1951).

The quantitative estimation of the urinary adrenaline and noradrenaline has in recent years gained diagnostic significance. ENGEL and v. EULER (1950) and several others have supplied evidence that in cases of pheochromocytoma the formation and excretion of these substances is greatly increased. The disease can be differentiated from other forms of arterial hypertension by determining the adrenaline and noradrenaline content in the urine or the blood.

The fluorescence reaction of adrenaline in alkaline solution was detected by LOEW already in 1918. Since then numerous attempts have been made to use this chemical reaction in quantitative studies of adrenaline in biological fluids. The reaction has been found to be very sensitive and specific for adrenaline and later also for noradrenaline. This reaction was used by PEKKARINEN and PITKÄNEN (1955 a, b) for estimations of adrenaline and noradrenaline in urine. It was the object of the present investigation to develop the fluorescence method further by finding the optimum conditions for the reaction. The purpose was also to determine the limitations and usefulness of the fluorescence method in scientific and clinical use. A further subject of study was the metabolic rôle of adrenaline in insulin-induced hypoglycaemia, as reflected in the urinary adrenaline and noradrenaline excretion.

DETERMINATION OF ADRENALINE AND NORADRENALINE IN THE URINE

Earlier Investigations

An extensive monograph on noradrenaline has been published quite recently (v. EULER 1956). It also contains a survey of the most widely used methods for the determination of noradrenaline in urine. These and other methods used in the study of urinary adrenaline and noradrenaline, including those which have subsequently been published, are reviewed below.

Chemical Methods

The pioneer attempts to demonstrate adrenaline in urine by chemical methods were qualitative. EMBDEN and FÜRTH (1903) isolated from rabbit urine a yellow compound after feeding large amounts of adrenaline. FALTA and IVCOVIC (1909) obtained a strongly positive reaction with ferric chloride in the urine of rabbits and dogs after feeding them adrenaline. Similar results were obtained by WEINSTEIN and MANNING (1937), who found that the injection of adrenaline into the rabbit resulted in an increase in the urinary excretion of phenolic substances.

It thus was possible at first to find adrenaline in the urine only after its administration into the test animal in large amounts. Gradually a technique was developed which made it possible to determine also the endogenous catechol amines. SHAW devised, in 1938, a method for the estimation of adrenaline in biological fluids in which for the first time the capacity of aluminium hydroxide to adsorb catechol amines in a slightly alkaline environment was applied to the purification of these substances. This discovery has been of importance also for the quantitative evaluation of the adrenaline and noradrenaline excretion. The aluminium hydroxide adsorption technique has since been widely used by several workers for the purification of adrenaline and noradrenaline in urine in both biological and chemical estimations (RICHTER 1940, BEYER and SHAPIRO 1945, HOLTZ *et al.* 1947, v. EULER and HELLNER 1951).

Using the adsorption technique of SHAW (1938), RICHTER (1940) demonstrated with various colour reactions the presence of exogenous adrenaline in human urine after the oral administration of large amounts of adrenaline. Most of the adrenaline excreted was in a bound form, probably conjugated with sulfuric acid. No free adrenaline was detected. A quantitative colorimetric estimation was also attempted. The method consisted in hydrolysis of the bound adrenaline by boiling with sulfuric acid, adsorption onto aluminium hydroxide and elution with an acid phosphate solution. After this treatment adrenaline was oxidised to a coloured form with iodine. The colorimetric evaluation was disturbed to a great degree by the many urinary pigments. The accuracy of the method was found to be satisfactory when the adrenaline excretion exceeded 1 mg. per hour. In the studies of RICHTER and McINTOSH (1941) the adrenaline ester was first purified by lead acetate adsorption. BEYER and SHAPIRO (1945) confirmed these studies by biological tests using the preparative treatment of RICHTER. The iodine oxidation was found suitable for the photometric determination of adrenaline. No basal excretion could be found in man and dog, but after oral administration large amounts of conjugated adrenaline were demonstrated in the urine. BEYER *et al.* (1951) applied this method to clinical studies. No catechol amines, however, could be detected in the urine in two cases of pheochromocytoma, whereas in a third case the urine, following a hypertensive crisis, contained conjugated adrenaline in a concentration of 0.45 mg. per 100 cc. of urine. BACQ *et al.* (1949), using the method of RICHTER, also found adrenaline in the urine of the dog after its oral administration.

In the method of SHAW (1938) the adrenaline content in the blood is estimated after the adsorption procedure by colorimetric measurement of the reduction of arsenomolybdic acid in alkaline solution. Previous treatment of the sample with alkali considerably intensifies the colour produced by adrenaline and this addition to the method is considered to be fairly specific for adrenaline. The various representatives of the biological catechol amines cannot be differentiated and the values therefore are expressed as adrenaline colour equivalents. This colorimetric method of SHAW has been applied by NUZUM and BISCHOFF (1953) to studies in urine. Colour-producing substances were found in the urine of normal subjects and of patients with arterial hypertension. The range of adrenaline excretion in normal subjects was 0.3—1.13 mg. in 12 hours, expressed as the adrenaline colour equivalent. The values exceed very many times the values obtained in urine by biological methods (v. EULER and HELLNER 1951, HOLTZ *et al.* 1947) and by the methods based on the fluorescence reaction (LUND 1952, PEKKARINEN and PITKÄNEN 1955 a), which seldom give excretion values as high as these even in cases of pheochromocytoma. RAAB and GIGEE (1954) also studied the urinary adrenaline excretion by the colorimetric method of SHAW. The

workers found no differences in the rate of adrenaline excretion between day and night, or in patients with emotional tension or arterial hypertension, in contrast to the biological results.

The colorimetric methods therefore appear to be rather unspecific in the evaluation of the adrenaline and noradrenaline content in the urine.

During the last few years, attempts have been made to use the fluorescence reaction of adrenaline and noradrenaline for quantitative determination in the urine. This reaction has been shown to be specific and as sensitive as the biological tests.

Most of the studies have concerned the use of this reaction for the determination of adrenaline in the blood (GADDUM and SCHILD 1933, v. HUEBER 1940, KALAJA and SAVOLAINEN 1941, LEHMANN and MICHAELIS 1943, JÖRGENSEN 1945). One of the difficulties in the earlier studies was the by-fluorescence that often totally concealed the fluorescence produced by adrenaline. Efforts were made to eliminate this by-fluorescence by precipitating the proteins by trichloroacetic acid (GADDUM and SCHILD 1933), by dialysing the plasma samples (v. HUEBER 1940, KALAJA and SAVOLAINEN 1941), or by using suitable specificity tests, such as formaldehyde treatment (LEHMANN and MICHAELIS 1943) and aeration of the plasma samples (JÖRGENSEN 1945). These various methods have given values for the plasma adrenaline content varying around 10 μg. per 100 cc. PEKKARINEN (1948) enhanced the specificity of these earlier methods by using aluminium hydroxide adsorption in combination with dialysis of the plasma sample. PEKKARINEN found that the adrenaline content of peripheral blood was below the range of the fluorescence reaction, being less than 0.5 μg. per 100 cc.

Owing to the two hydroxyl groups in the benzene ring, adrenaline and noradrenaline are readily oxidised. The oxidation occurs in alkaline solutions in the presence of oxygen, and in neutral or slightly acid environment with the aid of various oxidants such as iodine, silver or manganese dioxides. According to EHRLÉN, the oxidation begins with the formation of quinone, followed by an indole ring closure (1948). Red oxidation products, adreno- and noradrenochrome, are formed hereby. These compounds are readily oxidised further to melanine pigments. In alkaline solutions adreno- and noradrenochrome are transformed by intramolecular conversion to fluorescent substances called adrenolutine and noradrenolutine, respectively. These compounds also are oxidisable and the fluorescence is therefore labile. LUND (1949) succeeded in finding the optimum conditions for the fluorescence reaction. In neutral or slightly acid solutions adrenaline is oxidised by manganese dioxide to adrenochrome, which is then transformed to the fluorescent adrenolutine

by the addition of sodium hydroxide in high concentrations. Oxidation of adrenolutine is prevented by the simultaneous addition of ascorbic acid. Under these conditions noradrenaline also yields a fluorescence reaction, though only of about 50 per cent of the intensity produced by adrenaline (LUND 1950). These two catechol amines were differentiated by their dissimilar rates of oxidation at two different pH values.

The improved method introduced by LUND (1950) for the simultaneous determination of adrenaline and noradrenaline in blood may be regarded as an improvement in the determination of catechol amines. In addition to the development of the fluorescence reaction, extraction was also modified by the adsorption of adrenaline and noradrenaline onto Al_2O_3 in a column system. The values obtained by this method are definitely lower than those obtained by most of the other fluorescence methods. LUND was able to confirm the observation of PEKKARINEN (1948) that the adrenaline content of the peripheral blood is around the lower limit of sensitivity of the fluorescence reaction, and further to prove that also the noradrenaline content of the normal peripheral blood is negligible. This procedure is suitable also for adrenaline and noradrenaline estimation in urine (LUND 1952). The excretion rate for noradrenaline in normal subjects was between 40—85 μg. in 24 hours — values that are somewhat higher than those obtained by the biological method of v. EULER and HELLNER (1951). In four cases of pheochromocytoma there was an increased amount of adrenaline and noradrenaline in urine (1952). Using LUND's method GOLDENBERG *et al.* (1954), however, could not fully confirm his quantitative results in the urine of normal subjects. In their studies the fluorescence in the normal urine was frequently due for the greater part to non-specific fluorescent substances. However, they were able to distinguish the cases of pheochromocytoma with a high excretion rate from normal subjects.

GOLDENBERG *et al.* (1954) made extensive studies in order to work out a more specific photofluorimetric evaluation of the urinary catechol amines. The extraction procedure with alcohol-acetone in acid solution as used by v. EULER and HELLNER (1951) for the biological assay was found suitable when combined with the manganese dioxide treatment introduced by LUND (1950) for the development of the fluorescence reaction. However, also in this combination the various other fluorescent substances in urine caused great difficulties in the specific evaluation. In order to screen out the non-specific fluorescence it was necessary to use several filters. No absolute values were given by GOLDENBERG *et al.* for the normal rate of adrenaline and noradrenaline excretion, but in 16 cases of pheochromocytoma high readings were obtained. Differentiation between adrenaline and noradrenaline was rather difficult in this chemical modification also. A more specific estimation was accomplished by GOLDENBERG *et al.* (1954) after separating the catechol

amines from the other fluorescing substances by paper chromatography with 98 per cent phenol as the developing solvent.

BISCHOFF and GRAY (1956) carried out the manganese dioxide oxidation at pH 3 after purification of the urinary catechol amines by the adsorption method of SHAW. Only adrenaline is oxidised under these conditions. The values obtained also with this method were markedly higher than those obtained with biological methods.

In 1955 v. EULER and FLODING introduced a fluorimetric method for the estimation of adrenaline and noradrenaline in urine. In this procedure the aluminium oxide adsorption was adopted for the purification of these substances instead of the aluminium hydroxide earlier used in the biological estimation (v. EULER and HELLNER 1951). The elution was performed with a weak solution of sulfuric acid. In their study various agents were tested for the oxidation of adrenaline and noradrenaline. A weak solution of potassium ferric cyanide was suggested under these conditions. By this procedure it is possible to differentiate the two principal urinary catechol amines.

A quantitative fluorimetric and chromatographic method for catechol amine analysis in urine has recently been developed also by GOLDSTEIN and ABELIN (1956). As a further improvement the workers introduced the extraction of catechol amines from urine into butanol with the aid of o-nitrobenzoic acid.

The fluorescences in the eluates in the method of GOLDSTEIN and ABELIN were developed and stabilised by condensation of the catechol amines with ethylene diamine, as introduced by WEIL-MALHERBE and BONE (1952). This reaction has the advantage that, in addition to adrenaline and noradrenaline, hydroxytyramine also can be brought to a fluorescent state. The ethylene diamine reaction cannot, however, be applied to catechol amine estimations in urine without previous extensive purification of these substances (CHR. v. EULER *et al.* 1955). In addition to the use of chromatographic means (GOLDSTEIN and ABELIN 1956), an adequate purification can be accomplished also by an adsorption of the urinary catechol amines onto alumina, followed by an additional adsorption on cationic resins (WEIL-MALHERBE 1956). The values obtained with these methods in which the ethylene diamine reaction is used seem to correspond rather closely to the values obtained with the biological methods.

Biological Methods

In the first studies of the vasopressive substances in urine (ABELOUS and BARDIER 1909, BAIN 1910, BOHN and HAHN 1933, COESTER 1935, RISER *et al.* 1943) these principles were isolated by the use of procedures that presumably excluded the possibility that catechol amines would be

present in the final eluates. Only after the biological properties of adrenaline and noradrenaline were well established and suitable methods for their extraction from the urine were available could these substances be adequately determined also by biological means.

The purification procedure of RICHTER (1940) is suitable also for the biological assay of the urinary catechol amines. This was shown by BEYER and SHAPIRO (1945), who detected by this procedure a definite biological activity in human urine after the oral ingestion of adrenaline. BACQ *et al.* (1951), using the extraction procedure of RICHTER and McINTOSH (1941), found biological activity in the urine of the dog after an intravenous administration of large amounts of adrenaline. HOLTZ *et al.* (1947) were able by the same procedure to demonstrate also the presence of endogenous catechol amines in normal urine in man. The tests were carried out on cat's blood pressure and rabbit's intestine. This "Urosympathin" was later differentiated and found to consist almost entirely of noradrenaline (KRONEBERG and SCHÜMANN 1950). A quantitative estimation was also carried out. The 24-hour excretion of noradrenaline or adrenaline in normal subjects evaluated biologically by the preparative treatment of RICHTER and McINTOSH ranged between 100—150 μg.

The most widely used, specific and sensitive biological method for the determination of the naturally occurring adrenaline and noradrenaline in urine has been introduced by v. EULER and HELLNER (1951). In this procedure the catechol amines are first isolated from the urine samples by a modification of the aluminium hydroxide adsorption at pH 7.5, followed by elution with sulfuric acid. The salts are further precipitated out by treatment with alcohol-acetone and the tests are carried out on cat's blood pressure and hen's rectal coecum. The normal catechol amine excretion values for man obtained by this method are 4—8 μg. adrenaline and 20—40 μg. noradrenaline in 24 hours. This biological method has proved useful in both clinical and experimental work (v. EULER 1952, BURN 1953, ELMADJIAN *et al.* 1956 a, b) and it has also been applied to studies with experimental animals (SCHMITERLÖW 1951, v. EULER 1956). Later v. EULER and ORWÉN modified this method (1955). Instead of aluminium hydroxide, aluminium oxide was recommended for the adsorption of the catechol amines in urine, as used earlier by LUND (1952) and PEKKARINEN and PITKÄNEN (1955). The time-consuming treatment of the eluate with alcohol-acetone and the following evaporation can thus be eliminated. If a weak acid is used the eluate can be tested biologically as such.

KÄRKI (dissertation 1956) has also used the aluminium oxide adsorption and the elution with dilute sulfuric acid in the biological determinations. The eluate can be injected into the test animal after neutralisation. The mean normal excretion of free noradrenaline, estimated with this

method, was 20—26 μg. in 24 hours and that of adrenaline 4—6 μg. in 24 hours in adults.

An approach to the use of aluminium oxide adsorption followed by elution with a weak acid for the biological assay of urinary adrenaline and noradrenaline was also made by PEKKARINEN and PITKÄNEN in a preliminary report (1954). Trichloroacetic acid was used in the eluation. However, it turned out to be unsuitable since in samples with a low catechol amine content a depressor effect on the cat's blood pressure was sometimes obtained. PEKKARINEN and KÄRKI have later (personal communications, 1956) used in the biological adrenaline and noradrenaline estimations the aluminium oxide adsorption and elution with 1-N sulfuric acid. In the eluate, sulfuric acid is precipitated with calcium bicarbonate or with diluted sodium hydroxide to pH 4, followed by the removal of the calcium precipitate by centrifugation. The eluate is injected as such into the animal in the cat's blood pressure methods and neutralised further by very small amounts of sodium bicarbonate in the methods using hen's rectal coecum.

In urine samples where a high catechol amine content is encountered, as in cases of pheochromocytoma (CARL *et al.* 1951, WEIMAN *et al.* 1954, MOULTON and WILLOUGHBY 1955) and after an intravenous administration of noradrenaline into the test animal (FISCHER and LECOMPTE 1950 b), the biological assay can be accomplished by merely injecting untreated urine samples into suitably pretreated test animals. No reaction or a depressor action on the blood pressure is observed from normal samples, whereas samples with an elevated noradrenaline content produce a definite rise in the blood pressure. The lower limit of sensitivity of procedures of this kind is 100—200 μg. in 24 hours, and they can therefore be used for the diagnosis of pheochromocytoma (v. EULER 1952, MOULTON and WILLOUGHBY 1955). Since, on the other hand, the extraction technique for the biological determinations is specific and easy to carry out, it is generally preferable to use the adsorption and extraction procedures (personal communications by PEKKARINEN 1956, KÄRKI 1956).

Own Investigations

In 1955 a procedure was published (PEKKARINEN and PITKÄNEN) for a photofluorimetric evaluation of adrenaline and noradrenaline in urine. It was a modification of the method of LUND (1950) for the differential quantitation of adrenaline and noradrenaline in the blood. The aluminium oxide adsorption carried out by LUND in a column system was replaced by the use of a single adsorption of adrenaline and noradrenaline from

the urine specimen onto aluminium oxide by stirring. The change was made because of the problems encountered in the use of the column system due to precipitation of the urinary phosphates in an alkaline environment and to the formation of pores in the column *in vacuo*. The adsorption was followed by careful washing and by elution with 1-N oxalic acid, which precipitated the calcium salts and did not inhibit the fluorescence reaction of adrenaline and noradrenaline. The optimum conditions for the oxidation of adrenaline and noradrenaline by manganese dioxide and for the development of the fluorescence reaction after the addition of alkali were also discussed. For the sake of comparison the method is cited here briefly.

A 200 cc. specimen of urine is brought to pH 8.5 and the precipitated phosphates are centrifuged off.

Adrenaline and noradrenaline are adsorbed onto aluminium oxide by stirring for 5 minutes, followed by centrifugation.

The aluminium oxide precipitate is washed 3—5 times with distilled water and the catechol amines are eluated twice with 20 cc. of 1-N oxalic acid.

The pH of the eluate is raised to 6.3 and the eluate is diluted with 5—10 volumes of distilled water. Then 10 mg. of manganese dioxide is added to 6 cc. of this diluted eluate and mixed for 30 seconds and centrifuged.

To 2 cc. aliquots of the oxidised eluate is added 0.4 cc. of 5-N sodium hydroxide containing 200 mg. of ascorbic acid per 100 cc. and the intensity of the fluorescence reaction is determined and compared with adrenaline and noradrenaline standards added into aliquots of the eluate before oxidation. The blank value is obtained from an oxidised aliquot to which 0.4 cc. of sodium hydroxide without ascorbic acid has been added.

For a preliminary differentiation between adrenaline and noradrenaline it was suggested that the fluorescence reaction obtained with an addition of alkali without previous oxidation would be measured. In this condition adrenaline shows a much stronger fluorescence reaction than noradrenaline.

Although the quantitative estimation of adrenaline and noradrenaline by this method turned out to be relatively easy in the urine in cases of pheochromocytoma, in which the catechol amine content is high, various difficulties were experienced in the estimation of samples with a low adrenaline and nor-

adrenaline content, as in normal urine. The disadvantages of the method are discussed in the following paragraphs, with a description of the experiments conducted for their elimination. The intensities of the fluorescence have been determined in these studies with a "Farrand" fluorimeter, using Corning 5860, double thickness, as the primary filter and Corning 3389, double thickness, as the secondary filter.

1. Inhibition of the Fluorescence Reaction

The strong inhibition of the fluorescence of undiluted eluates of different urine samples, as estimated with standard additions of adrenaline and noradrenaline, was a source of great difficulties and gave reason to study this phenomenon further.

For the elimination of the inhibition high dilutions of the eluate were suggested (PEKKARINEN and PITKÄNEN 1955). Nevertheless a definite inhibition was still observed in several samples. It was characteristic that the inhibition in the urine was only partially abolished by a stronger oxidation with manganese dioxide. In extreme cases the inhibition was strong enough to compel the performance of a series of oxidations with different amounts of manganese dioxide to find the optimum conditions for the fluorescence reaction and to obtain measurable readings. Furthermore, it is probable that in samples in which a weak adrenaline and noradrenaline fluorescence was encountered the part played by the non-specific fluorescent substances in the readings was correspondingly increased. It was, therefore, deemed advisable to make arrangements to eliminate this disadvantage.

The fluorescent compounds adreno- and noradrenolutine are intermediate products in the oxidation of adrenaline and noradrenaline to melanine pigments. The development of the fluorescence is, therefore, an equilibrium reaction which is labile in nature and easily disturbed by various agents. The fluorescence reaction of adrenaline is shown to be largely dependent on the ionic environment of the solution (HELLER *et al.* 1950, RIBEIRO and CARDOSO 1950, TRAUTNER and MESSER 1952). The oxidation is more rapid in a phosphate than in a carbonate

buffer solution (CHAIX and PALLAGET 1953) and is inhibited by various amino acids and proteins (BOUVET 1949 a, b, MYLON and ROSTON 1953) and naturally by various reducing substances. Aluminium and zinc ions enhance the isomerisation of adrenochrome to the fluorescent adrenolutine (FISCHER *et al.* 1950). In the present studies it was observed that the fluorescence reaction is also dependent on the acid used for the elution.

The use of 1-N oxalic acid for the elution has been suggested (PEKKARINEN and PITKÄNEN 1955). As adrenaline and noradrenaline can be eluated from aluminium oxide already when the acidity has been brought to about pH 4 (LUND 1950), it seems unnecessary to use such a strong acid for this purpose. It may be assumed, on the contrary, that such an acid dissolves various ions and possibly also various fluorescent substances more than a more dilute acid. It was difficult in the present study, however, to obtain a strong fluorescence reaction of adrenaline and noradrenaline in urine eluates made with dilute acids (0.1—0.2-N), and the recovery was not quantitative. In several samples the intensity of the fluorescence in the standards remained less than 20 per cent of the intensity obtained in eluates made with 1-N oxalic or sulfuric acids. It is possible that these more concentrated acids dissolved some of the aluminium oxide, as aluminium salts are known to enhance the fluorescence reaction (FISCHER *et al.* 1950). The use of a very diluted acid in the eluation therefore does not seem advisable.

As nothing is known concerning the substances which produce the inhibitory effect in the urine samples, the adjustments made for its elimination can necessarily be secondary only. A dilution of the eluate is the only means known to reduce effectively the strength of the inhibition (PEKKARINEN and PITKÄNEN 1955). Since in the eluate diluted with ten volumes of distilled water, as suggested earlier, some inhibition was encountered, a still higher dilution was taken into use. In a fifteen-fold dilution of the eluate a strong fluorescence reaction was constantly obtained in the standards of adrenaline and noradrenaline and its intensity was almost uniform in the different samples. This additional dilution in a few of the samples increased the intensity of the

fluorescence of standards by 50 per cent, making it as strong as that obtained under optimum conditions in pure buffer solutions.

As a further adjustment the urine specimens were diluted to correspond to an excretion of 1500 cc. in 24 hours. This adjustment made the various samples more uniform.

2. Precipitation in the Eluate

When a strong acid was used, the eluate contained various salts that had a tendency to precipitate when the pH of the eluate was raised. This was true also after the dilution of the eluate. The precipitate was formed when the pH was elevated to 5 and it was only partially dissolved at pH values over 8. As the fluorescence reaction was measured in an alkaline environment, this precipitate often interfered considerably. A part of the precipitate may be mucin-like substances and a part consists of dissolved aluminium oxide. A considerable proportion is due to urinary phosphate that is precipitated at the adsorption stage at pH 8.5 and remains among the aluminium oxide particles. Washing with water seldom dissolved them totally. This urine precipitate was observed to partly adsorb adrenaline and noradrenaline and it therefore could not be filtered off before the addition of aluminium oxide. The dilution of the urine specimens with 4 volumes of water and the addition of 1 per cent sodium citrate to bind the calcium ions before the pH of the urine samples was brought to 8.5 prevented effectively the precipitation of phosphate. No undue precipitation has been encountered after this adjustment in the later stages of the procedure.

3. Specificity of the Method

The mean normal adrenaline and noradrenaline excretion determined by the fluorescence method was shown to be 81 μg. in 24 hours (PEKKARINEN and PITKÄNEN 1955 b). It exceeded clearly the mean value obtained by the biological methods (v. EULER and HELLNER 1951). It is to be considered possible, therefore, that in spite of the adsorption and vigorous washing

some non-specific substances participate in the fluorescence in addition to adrenaline and noradrenaline. Evidently these substances may be stabilised by ascorbic acid in an alkaline solution and their fluorescence fades in those specificity tests in which the ascorbic acid is omitted.

A. Treatment of the Eluate with Butyl Alcohol

The extracts obtained by the biological method of v. EULER and HELLNER (1951), which consists in an aluminium hydroxide adsorption and a salting-out with alcohol-acetone, contain substances that interfere with the fluorimetric evaluation of urinary adrenaline and noradrenaline (GOLDENBERG *et al.* 1954, PITKÄNEN, unpublished). This suggests that these non-specific substances are, at least in part, soluble in lipoid solvents. This opinion was supported also by the preliminary experiments of the present author, in which the catechol amines of the urine eluates were run on paper chromatograms in a butyl alcohol

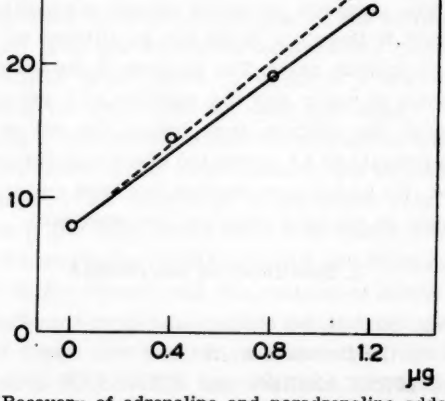

Figure 1. Recovery of adrenaline and noradrenaline added to eluates before extraction with butyl alcohol. Abscissa: amount of adrenaline and noradrenaline (1 : 1) added to 1 cc. of eluate. Ordinate: intensity of fluorescence. Dotted line: 100 per cent recovery. Solid line: recovery obtained.

Figure 2. Effect of butyl alcohol treatment in five urine specimens (I—V). B=blank. S=sample. St=sample with standard noradrenaline addition (2.5 μg. per 100 cc.). Ordinate: intensity of fluorescence. Total column: fluorescence without butyl alcohol treatment. Shaded portion: fluorescence after butyl alcohol treatment.

system. Strongly fluorescent substances were found to run at a more rapid rate than catechol amines. On the other hand, the solubility of adrenaline and noradrenaline in various lipoid solvents is known to be negligible at an acid pH. Experiments were conducted, therefore, in which the acid eluates were extracted with various water-insoluble lipoid solvents. Of the various agents tested, ether and chloroform were incapable of removing any of the non-specific fluorescence. The water-insoluble alcohols, on the other hand, reduced it effectively.

In the experiments for which the results are shown in figures 1 and 2 and in table 1, the urine eluates were made in 1-N sulfuric acid. The acid eluates were extracted by vigorous shaking with an equal volume of normal butyl alcohol. After the emulsion had been allowed to break up into phases, the butyl alcohol layer was removed by suction through a thin pipette. The eluates were diluted with 15 volumes of distilled water, the pH was raised to 6.3 and the fluorescences were developed. The adrenaline and noradrenaline standards were added before the extraction with butyl alcohol in amounts to make a final concentration of 2.5 μg. per cent (figure 2, table 1).

Adrenaline and noradrenaline remained almost completely in the acid phase when the eluate was extracted with butyl

Table 1. Effect of butyl alcohol treatment on the values obtained for the adrenaline and noradrenaline excretion in 24 hours.

No treatment	With treatment
200	62
310	160
340	98
180	31
185	22
144	18
250	10
50	11
Mean=209 μg.	Mean= 54 μg.

alcohol (figure 1). The butyl alcohol extraction reduced effectively the side fluorescence of the sample and the blank, as demonstrated in the five typical examples shown in figure 2. The intensities of the fluorescence were sometimes reduced (exx.: columns II and IV) to almost 10 per cent of the intensities obtained without the butyl alcohol treatment. Also the intensity of the fluorescence in the standard was often reduced owing to the lowering of the non-specific fluorescence. The fluorescence of the adrenaline and noradrenaline standards often was stronger after treatment with butyl alcohol, perhaps due to the stabilising effect of butyl alcohol. Butyl alcohol is a reducing substance, it stabilises, for instance, adrenochrome and noradrenochrome in slightly alkaline solutions (PEKKARINEN, unpublished). The changes in the fluorescence of the blank and sample due to the butyl alcohol extraction greatly altered the values obtained for the adrenaline and noradrenaline excretion. Some of the most distinct alterations are summarised in table 1. The difference in the values obtained with and without the butyl alcohol extraction is to be considered to be due to non-specific fluorescence.

B. Reaction Blank

The fluorescent compounds, adreno- and noradrenolutine, tend to be rapidly destroyed in alkaline solution in the absence of stabilising agents. This phenomenon was applied by LUND (1950) to the blank reaction in which an aliquot of the sample was treated with manganese dioxide and alkalinised, but in which

the addition of ascorbic acid was omitted. The reading of an aliquot stabilised by ascorbic acid was compared with this blank. This procedure was adapted by GOLDENBERG *et al.* (1954) and PEKKARINEN and PITKÄNEN (1955) to the photofluorimetric evaluation of adrenaline and noradrenaline in urine.

a. Use of Formaldehyde as Blank

The oxidative destruction of the fluorescence of adrenaline and noradrenaline must be regarded as too unspecific for use in the blank. In earlier investigations reported in the literature, substances were therefore sought that would more specifically destroy the fluorescence of adrenaline-like substances. Formaldehyde was the most widely used substance of this kind (LEHMANN and MICHAELIS 1943, JÖRGENSEN 1945).

The following experiments were carried out with samples eluated with 1-N sulfuric acid. The eluates were diluted 15-fold without previous extraction with butyl alcohol. After the dilution and the raising of the pH of the eluate to 6.3, formaldehyde was added to make a concentration of 1 per cent. Both the samples and the aliquots treated with formaldehyde were oxidised with equal amounts of MnO_2, alkalinised, and stabilised with equal amounts of ascorbic acid. The results of these experiments were similar in the different samples. The four examples,

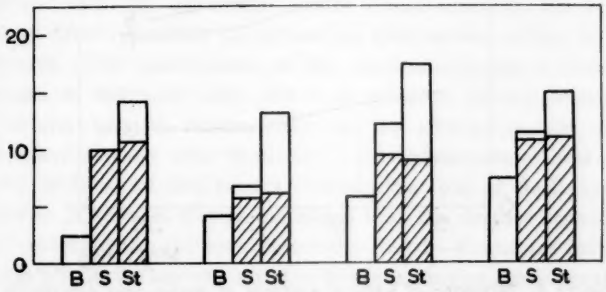

Figure 3. Effect of formaldehyde in 4 urine specimens. B=blank. S=sample. St=standard addition of noradrenaline (2.5 μg. per 100 cc.). Total column: fluorescence without formaldehyde treatment. Shaded portion: fluorescence after formaldehyde treatment.

demonstrated in figure 3, are typical. The blank value (B) obtained with the omission of ascorbic acid and without the formaldehyde treatment are included for the sake of comparison. The final noradrenaline concentration in the standards was 2.5 μg. per 100 cc. The results in figure 3 show that formaldehyde was able to abolish also the fluorescence of noradrenaline when added before the oxidation with manganese dioxide. However, the fluorescence in the formaldehyde-treated aliquots as indicated by the dark columns was stronger than in the blank, although in both the fluorescence due to adrenaline and noradrenaline was abolished. In the experiment demonstrated in figure 4 the fluorescence in the formaldehyde-treated aliquot also was stronger than in the blank (without ascorbic acid addition). In this experiment, the aliquots were treated with various (5—35 mg.) amounts of MnO_2. Even a strong oxidation was not capable of counteracting the effect of formaldehyde on the fluorescence of the sample and the standard addition. The difference in fluor-

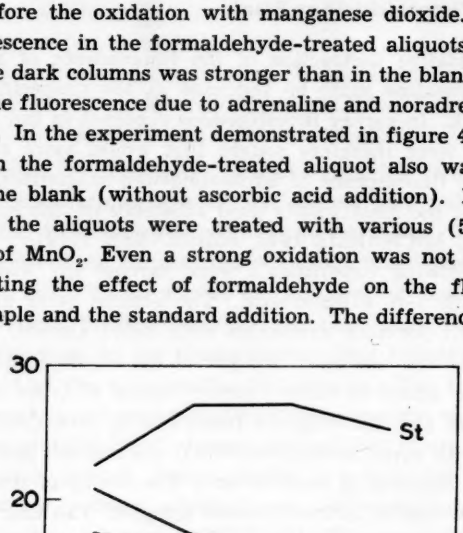

Figure 4. Influence of various amounts of manganese dioxide on the effect of formaldehyde. B=blank. S=sample. St=standard addition of noradrenaline (4 μg. per 100 cc.). Ordinate: intensity of fluorescence. Abscissa: amount of manganese dioxide added to 5 cc. of diluted eluate at pH 6.3. F=formaldehyde-treated sample and standard.

escence in the formaldehyde-treated aliquot and in the blank made without ascorbic acid is evidently due to substances other than adrenaline and noradrenaline. The own fluorescence of formaldehyde was negligible.

Especially the studies on butyl alcohol extraction and in part also those on the formaldehyde treatment strongly suggest that the urine eluate contains non-specific substances that react in the blank without ascorbic acid as introduced by LUND. The use of the butyl alcohol extraction therefore seems to be well established in order to secure more specific fluorescence values for the urinary adrenaline and noradrenaline content. Since formaldehyde is a highly reactive compound it can be postulated that treatment with this substance may form other fluorescent substances in the blank and its use was therefore abandoned.

b. Addition of Ascorbic Acid in the Blank

Due to the labile nature of adreno- and noradrenolutine the fluorescence of adrenaline and noradrenaline disappears rapidly in alkaline solution unless stabilising substances are present. In most of the manganese dioxide-treated urine eluates also, the fluorescence due to adrenaline and noradrenaline was negligible already within 3—4 minutes after the addition of alkali if the ascorbic acid was omitted. This aliquot has therefore been used as blank in this method. In the present studies, however, it was observed that the behaviour of the fluorescence in the blank differed from that due to adrenaline and noradrenaline in the standards. The fluorescence of the blank continued to steadily decrease in intensity after the 3—4 minutes' period, although all of the specific fluorescence due to adrenaline and noradrenaline already was destroyed. This phenomenon was observed in most of the blanks, though the rate of decline was variable. It seemed likely, therefore, that the further fading of the fluorescence in the blank after the first 3—4 minutes following the alkalinisation was not due to adrenaline or noradrenaline but was an unspecific phenomenon. The use of the aliquot without ascorbic acid as blank, therefore, is necessarily somewhat arbitrary.

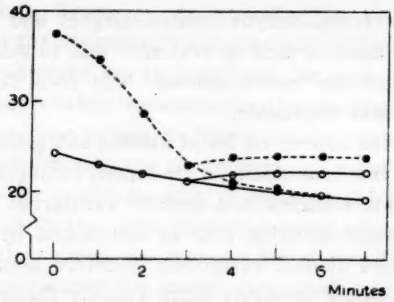

Figure 5. Decline of fluorescence after alkalinisation in absence of ascorbic acid and effect of addition of ascorbic acid. Ordinate: intensity of fluorescence. Solid line: sample. Dotted line: noradrenaline standard (2.5 μg. per 100 cc.).

An addition of ascorbic acid prevented this non-specific decrease in the fluorescence of the blank without restoring any of the fluorescence due to adrenaline or noradrenaline. It seemed advisable, therefore to add ascorbic acid to the blank aliquot 3—4 minutes after the alkalinisation. Owing to its own fluorescence and to impurities, ascorbic acid increased slightly the fluorescence of the sample and blank.

This phenomenon is demonstrated in figure 5. The gradual, continuous decline in the fluorescence of the blank after addition of the alkali (solid line) is clearly visible in this experiment, as is also the prevention of this decline by ascorbic acid (20 mg. per cent). The fluorescence in the noradrenaline standard (2.5 μg. per cent, dotted line) is destroyed within 3—4 minutes and the addition of ascorbic acid does not restore it.

It may be postulated, however, that some non-specific decline in the fluorescence in the blank occurs also within the first 3—4 minutes after alkalinisation. To what extent this is true could not be stated because of the simultaneous decrease in the adrenaline and noradrenaline fluorescence, but it is likely that as a consequence of this decrease the values obtained with this method will somewhat exceed the actual values for adrenaline and noradrenaline excretion.

After the period of 3—4 minutes the fluorescence of noradrenaline was restored by ascorbic acid in most blanks to a

negligible degree only. In a few of the blanks, however, the destruction was not complete in the course of this interval and up to 15 per cent of the fluorescence of noradrenaline was sometimes restored by the addition of ascorbic acid. This error is eliminated if the stabilisator is added after a longer interval, but, on the other hand, this increases the error due to the non-specific fluorescence.

C. Hydroxytyramine

In the present studies the standard amounts of hydroxytyramine added to the urine eluates yielded a fluorescence reaction of varying but negligible intensity in the different urine samples. The intensity of the hydroxytyramine fluorescence in the 21 samples tested varied from 0 to 6 per cent, mean 3.5 per cent, of the noradrenaline fluorescence reaction. Hydroxytyramine is also adsorbable onto aluminium oxide and is eluated with 1-N sulfuric acid and remains in the acid phase in the butyl alcohol extraction.

4. Differentiation of Adrenaline and Noradrenaline

Under optimum conditions, adrenaline and noradrenaline can be differentiated in several ways, all of which are based on the different sensitivity of these two catechol amines to oxidation.

LUND (1950), using acetic acid eluates for the study of adrenaline and noradrenaline in blood, made the differentiation after oxidation with MnO_2 at two different pH values. Both adrenaline and noradrenaline were oxidised at pH 6 and yielded a fluorescence reaction after an addition of alkali and ascorbic acid. At pH 3 only adrenaline but not noradrenaline was quantitatively oxidised, enabling the differentiation of these two catechol amines by comparison of the two values. In the present studies to adopt this procedure for the differentiation in urine it was found, however, that the behaviour of adrenaline and noradrenaline against oxidation in the urine samples eluated with 1-N sulfuric acid was quite different from that in blood. In the experiment shown in figure 6, the oxidations were carried

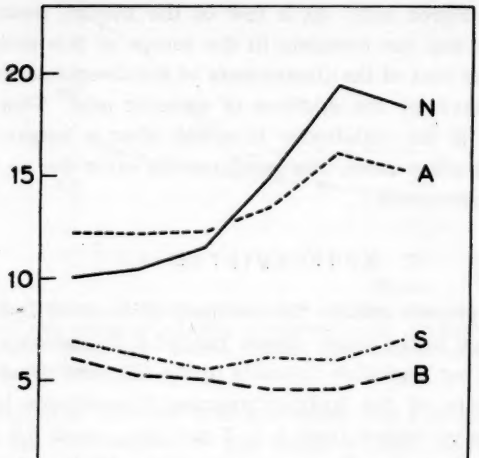

Figure 6. Effect of pH in eluate. B=blank. S=sample. N=noradrenaline standard (5 μg. per 100 cc.). A=adrenaline standard (5 μg. per 100 cc.). Ordinate: intensity of fluorescence.

out with equal amounts of MnO_2 at different pH values in sulfuric acid urine eluates after butyl alcohol extraction and dilution of the eluate (1 : 15). The fluorescence intensities in the adrenaline and noradrenaline standards were low when the oxidation was carried out at low pH values and did not differ to such a degree as to permit differentiation. Furthermore, at low pH values the non-specific substances evidently interfered considerably. Differentiation in the urine eluates when the oxidation was carried out at two different pH values was therefore found to be difficult. In their studies with urine GOLDENBERG *et al.* (1954) also found differentiation by this means infeasible.

After an addition of alkali, adrenaline, unlike noradrenaline, yields a fluorescence reaction also without preceding treatment with oxidative agents and without the presence of ascorbic acid (LOEW 1918, GADDUM and SCHILD 1933, KALAJA and SAVOLAINEN 1941, PEKKARINEN 1948). This reaction, used

earlier in experiments with blood, is known to be of the order of only one-fourth of the maximum adrenaline fluorescence obtained by manganese dioxide oxidation and stabilisation with ascorbic acid, it is also known to fade rapidly (LUND 1950). The use of this fluorescence reaction of adrenaline for the differentiation between adrenaline and noradrenaline in the urine eluates was suggested especially in cases of pheochromocytoma (PEKKARINEN and PITKÄNEN 1955). The reaction, however, is not sufficiently sensitive to detect adrenaline in normal urine, all the more so as high dilutions of the eluates were employed in the present work.

Adrenolutine, the fluorescent form of adrenaline, is a more labile compound than noradrenolutine in alkaline solution unless a stabilising substance such as ascorbic acid is present. In the studies on blood by LUND (1950), for instance, the fluorescence of the noradrenaline standard in the blank was visible for half

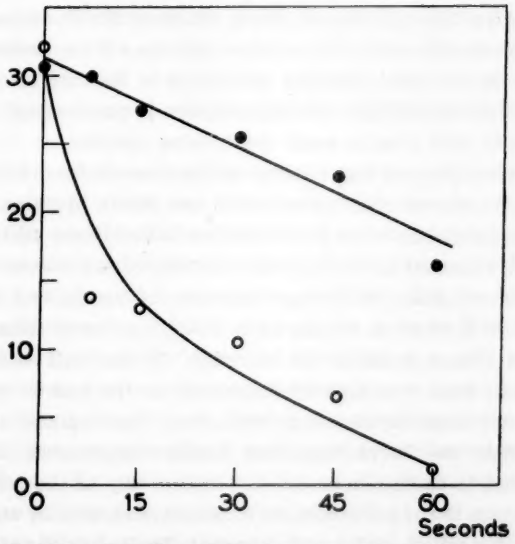

Figure 7. Rate of decline of fluorescence after addition of alkali in absence of ascorbic acid. Ordinate: intensity of fluorescence. ●=noradrenaline standard (10 μg. per 100 cc.). ○=adrenaline standard (10 μg. per 100 cc.). pH of the eluate = 6.3.

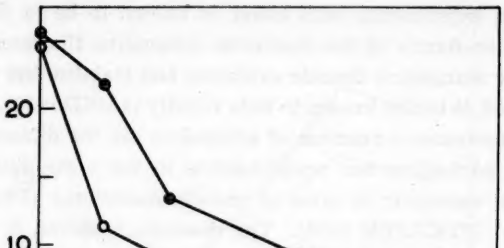

Figure 8. Same as figure 7. ●=sample. ○=adrenaline standard (7 μg. per 100 cc.). +=noradrenaline standard (7 μg. per 100 cc.).

an hour after the addition of alkali, whereas the fluorescence of adrenaline disappeared within a few minutes. It has been shown in the present studies that this difference in the rate of destruction of adrenolutine and noradrenolutine is pronounced also in the sulfuric acid eluates made from urine specimens.

This phenomenon was studied in the present investigation in a series of experiments, two of which are shown in figures 7 and 8. The experiments were performed in 1-N sulfuric acid eluates after butyl alcohol extraction and dilution of the eluate (1 : 15) and after oxidation with equal amounts of manganese dioxide. Adrenaline showed in blanks after alkalinisation a fluorescence that was almost maximal in intensity. In one-half minute the fluorescent form was already destroyed for the greater part and only a moderate fluorescence remained. One minute after alkalinisation the fluorescence had totally disappeared. Ascorbic acid added to these blanks did not restore any of the adrenaline fluorescence but it prevented its further destruction by stabilising that portion which still was fluorescent. The behaviour of the noradrenaline fluorescence in the blank was similar, with the difference that the destruction rate was less rapid, the noradrenaline

fluorescence disappearing completely only after 3—4 minutes. Ascorbic acid had a stabilising effect also on the noradrenaline fluorescence without restoring any of the fluorescence which had been destroyed.

This difference between adrenaline and noradrenaline in the rate of destruction of fluorescence in blanks offered the best possibilities for a differentiation under these experimental conditions. For this purpose it was advisable to have two blanks. Ascorbic acid was added to one of the blanks 45 seconds after the alkalinisation. By this time most of the adrenaline fluorescence had already faded, whereas the noradrenaline fluorescence still was almost maximal. Ascorbic acid was added to the other blank 4 minutes after the alkalinisation. The fluorescence of both adrenaline and noradrenaline in the blank had disappeared by this time.

5. Other Modifications

The high dilution of the eluate made it possible to perform the analyses with a smaller volume of urine. The analysis may well be carried out with 12.5 cc. instead of the 200 cc. of urine used in the earlier experiments (PEKKARINEN and PITKÄNEN 1955).

In that work the adsorption of adrenaline and noradrenaline onto aluminium oxide was carried out with the aid of a stirrer followed by centrifugation. This was found to be practical, since large amounts of urine were analysed. In the present modification using 12.5 cc. of urine it was shown, however, that the adsorption was as effective at pH 8.5 if the samples were shaken by hand for 4—5 minutes. Centrifugation thereafter was also rendered unnecessary by merely allowing the samples to stand for a few minutes after being shaken. During the standing of the sample the aluminium oxide particles separated from the solution and settled down. To avoid undue oxidation of adrenaline and noradrenaline during this period when the pH of the samples was high, about 20 mg. of ascorbic acid was added to the samples.

After the adsorption of adrenaline and noradrenaline at pH 8.5, aluminium oxide was washed once with distilled water by

shaking by hand and allowing the particles to separate. As the supernatant was decanted off, a considerable portion of distilled water remained among the aluminium oxide particles and this increased the volume of the eluate. The whole procedure was changed, therefore, to occur in a separatory funnel with a tight glass wool plug packed in the bottom. The portion of water remaining among the particles after decanting was drained by suction through this glass wool layer before the elution.

The 1-N oxalic acid used earlier (PEKKARINEN and PITKÄNEN 1955) for eluation has been replaced by 1-N sulfuric acid. The main reason for this change was the scantier recovery of adrenaline and noradrenaline in the butyl alcohol extraction in the eluates for which oxalic acid was used. Like oxalic acid, sulfuric acid precipitates the calcium salts.

6. Optimal Conditions for the Fluorescence Reaction

The modifications in the method, described in sections 1—5, have altered the conditions under which the fluorescence of adrenaline and noradrenaline is developed. Consequently the conditions influencing the oxidation and the isomerisation to the fluorescent form that previously were found suitable could not be applied directly. In the following experiments these conditions, *i.e.*, the optimum pH, ionic concentration, amount of oxidant and time of oxidation, have been controlled. Experiments were thus conducted in eluates made with 1-N sulfuric acid followed by extraction with butyl alcohol. The oxidation was carried out by shaking the aliquots in centrifugal tubes with manganese dioxide, followed by centrifugation. Unless stated otherwise, the pH of the eluate was raised to pH 6.3 with sodium acetate, the eluates were diluted with 15 volumes of distilled water and adrenaline and noradrenaline in standards were added to make a final concentration of 5 μg. per 100 cc., 5 mg. of MnO_2 was used for each 5 cc. of diluted eluate. The oxidation time was 4 minutes. Ascorbic acid was added to 5-N sodium hydroxide just prior to use to make a final concentration in the eluates of 20 mg. per cent.

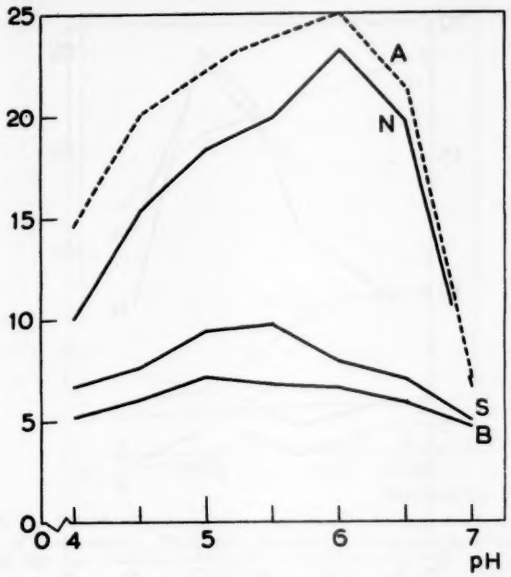

Figure 9. Effect of pH. Ordinate: intensity of fluorescence. B=blank. S=sample. A=adrenaline standard (5 μg. per 100 cc.). N=noradrenaline standard (5 μg. per 100 cc.).

A. pH of the Eluate

The pH range 6.0—6.5 was optimal for the oxidation of adrenaline and noradrenaline with MnO_2 (figures 6, 9 and 10). At a more alkaline reaction a precipitate was formed in the eluate onto which adrenaline and noradrenaline were adsorbed and thus escaped oxidation. When the oxidation was carried out at a more acid reaction a decrease in the intensity of the fluorescence of adrenaline and noradrenaline standards was also noticed. The decrease in the fluorescence of both these substances was pronounced. In spite of the decrease in the fluorescence of the standards at the lower pH values, the difference between the sample and blank did not decrease proportionally. This can be assumed to indicate the presence of interfering non-specific fluorescent substances. Their influence is naturally lowest at

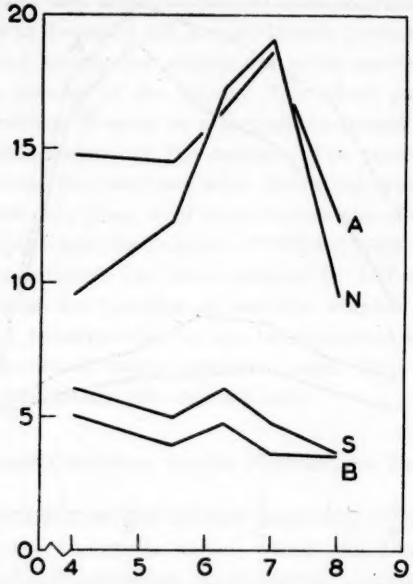

Figure 10. Effect of pH. Test conditions as in figure 9.

pH 6.0—6.5, where the adrenaline and noradrenaline fluorescence is maximal.

B. Concentration of the Buffer Solution

The concentration of the sodium acetate buffer solution in the eluate had a definite effect on the fluorescence of adrenaline and noradrenaline standards, as demonstrated in figure 11. At low acetate buffer concentrations (up to 0.75 M) a precipitate tended to form in the eluate at pH 6 which was capable of adsorbing catechol amines. The low intensity of the fluorescence of the adrenaline and noradrenaline standards then was evidently due to this effect. The best fluorescence of the noradrenaline and adrenaline standards was obtained with 2-M sodium acetate buffer.

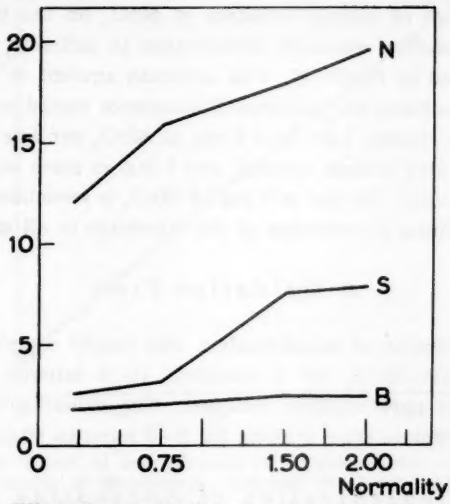

Figure 11. Effect of concentration of sodium acetate buffer. Ordinate: intensity of fluorescence. B=blank. S=sample. N=noradrenaline standard (5 μg. per 100 cc.).

C. Amount of Oxidant

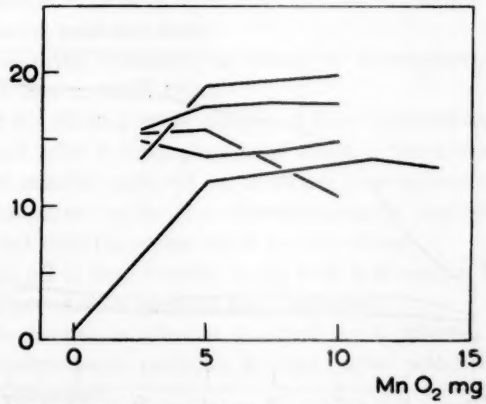

Figure 12. Effect of varying doses of manganese dioxide in 5 urine specimens. Final concentration of noradrenaline standard=5 μg. per 100 cc. Ordinate: intensity of fluorescence. Abscissa: amount of MnO_2 added to 5 cc. of diluted eluate.

The effect of various amounts of MnO_2 on the intensity of the noradrenaline standard fluorescence in different samples is demonstrated in figure 12. The optimum amount of manganese dioxide to achieve the maximal fluorescence varied considerably in different eluates. Less than 5 mg. of MnO_2 per 5 cc. of diluted eluate was very seldom optimal, and 5 mg. or more was required in most samples. The use of 5 mg. of MnO_2 is recommended, since it gave a strong fluorescence of the standards in all samples.

D. Oxidation Time

The oxidation of noradrenaline was nearly complete after a shaking with MnO_2 for 3 minutes. In 8 minutes no undue oxidation of noradrenaline occurred. The oxidation of adrenaline was complete after shaking for 2—3 minutes (figure 13).

E. Concentration of Adrenaline and Noradrenaline

The concentration curve for adrenaline and noradrenaline was linear to at least 40 μg. per cent in the final solution (figure 14).

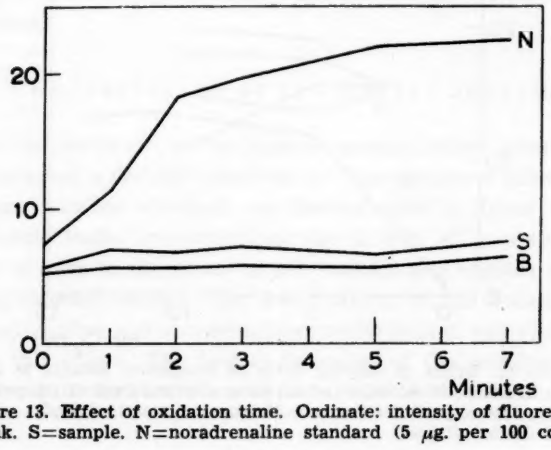

Figure 13. Effect of oxidation time. Ordinate: intensity of fluorescence. B=blank. S=sample. N=noradrenaline standard (5 μg. per 100 cc.).

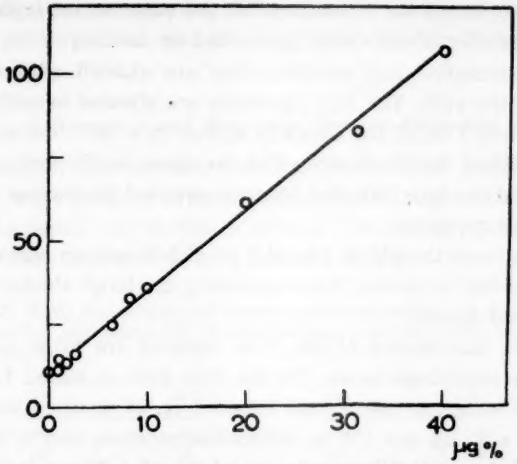

Figure 14. Effect of concentration of adrenaline and noradrenaline. Ordinate: intensity of fluorescence. Abscissa: Final concentration of adrenaline and noradrenaline (1 : 1).

7. The Method.

On basis of the experiments described above, the method introduced by PEKKARINEN and PITKÄNEN (1955) for the determination of urinary adrenaline and noradrenaline was given the following modified form.

1. The urine specimen is diluted to correspond to an excretion of 1500 cc. in 24 hours.

2. Of the diluted urine sample, 12.5 cc. is placed in a separatory funnel with a tight glass wool layer in the bottom. About 20 mg. of ascorbic acid, 0.5 cc. of 50 per cent sodium citrate, 1.5 g. of aluminium oxide (for chromatographic analysis, Savory Moore) and distilled water *ad* 50 cc. are added.

3. The pH is then brought to 8.5 with 2-N sodium hydroxide, followed by vigorous shaking for 5 minutes.

4. The sample is allowed to stand for 4 minutes to permit the aluminium oxide particles to settle, after which the supernatant is decanted.

5. The aluminium oxide particles are washed with 50 cc. of distilled water by shaking thoroughly. The sample is again

allowed to stand for 3 minutes and the supernatant is decanted. The remainder of the water is removed by suction.

6. Adrenaline and noradrenaline are eluated with 5 cc. of 1-N sulfuric acid. The Al_2O_3 particles are allowed to settle down.

7. Then 1 cc. of the eluate is shaken in a test tube with 1 cc. of redistilled butyl alcohol. The emulsion is allowed to break down and the butyl alcohol layer is removed by suction through a thin glass pipette.

8. To raise the pH to 6.3, 14.2 cc. of 2-N sodium acetate solution is added to the test tube containing the butyl alcohol-treated eluate and mixed.

9. Of this diluted eluate 5 cc. samples are taken into each of three centrifuge tubes. To the first tube is added 1.5 cc. of distilled water, to the second tube 0.5 cc. of distilled water and 1 cc. of a 25 μg. per 100 cc. adrenaline solution, and to the third tube 0.5 cc. of distilled water and 1 cc. of a 25 μg. per 100 cc. noradrenaline solution.

10. To each of the three tubes 5 mg. of freshly washed MnO_2 is added, the whole is shaken for 3.5 minutes and then centrifuged.

11. Three samples of 2 cc. are taken from each centrifuge tube.

To one of the 2 cc. samples from each tube 0.4 cc. of 5-N sodium hydroxide containing 100 mg. per 100 cc. ascorbic acid is added.

To the second sample 0.36 cc. of 5-N sodium hydroxide is added, followed 45 seconds later by an addition of 0.04 cc. of 1 per cent ascorbic acid solution.

In the third sample the addition of sodium hydroxide (0.36 cc., 5-N) is followed 4 minutes later by the addition of ascorbic acid (0.04 cc., 1 per cent).

The adrenaline and noradrenaline conjugates can be split and estimated by keeping the urine samples for 20 minutes in a boiling water bath at pH 1 (v. EULER and HELLNER 1951). The analysis is then carried out as above.

The Farrand photoelectric fluorimeter is suitable for the determination of the low intensities of fluorescence in these

diluted specimens. Corning 5860 (double thickness) was used as the primary filter and Corning 3389 (double thickness) as the secondary filter.

8. Accuracy and Recovery. Normal Excretion

To control the accuracy of the method, 18 simultaneous analyses were made of samples taken from a single urine specimen. The results are shown in table 2. The statistical treatment of the results is presented in a separate chapter.

Table 2. Total adrenaline and noradrenaline excretion (μg. in 24 hours) and adrenaline percentage in 18 simultaneous analyses made in a single urine specimen.

μg.	%	μg.	%
55	0	78	0
83	0	81	0
52	55	41	55
45	25	39	50
35	75	39	45
50	30	62	45
56	50	56	50
33	33	57	25
54	25	58	25
		Mean=54	Mean=33

The recovery of adrenaline and noradrenaline in the course of these experiments was repeatedly controlled with the aid of adrenaline and noradrenaline standards added to the specimens before the adsorption with aluminium oxide at pH 8.5. The results of the controls are summarised in table 3. The mean recovery of noradrenaline was 69.5 per cent, and of adrenaline 76.1 per cent.

Table 3. Recovery of adrenaline and noradrenaline.

Per cent	45-49	50-59	60-69	70-79	80-100	
Number of analyses	4	10	24	16	8	Noradrenaline
	—	1	4	10	5	Adrenaline

The 24-hour excretion of adrenaline and noradrenaline in 16 healthy subjects is shown in table 4. The mean total adrenaline

Table 4. Normal adrenaline and noradrenaline excretion (μg. in 24 hours) and adrenaline percentage in sixteen healthy adults.

Unhydrolysed		Hydrolysed	
μg.	%	μg.	%
26	30	70	35
49	40	72	30
42	15	53	15
39	35	77	45
46	25	90	10
39	10	73	10
33		73	
32		129	
36		102	
90	50		
80	25		
100	60		
80	40		
75	30		
45	20		
80	40		
Mean=54	Mean=33	Mean=82	

and noradrenaline excretion was 54 μg. in 24 hours, with an adrenaline percentage of 33 in unhydrolysed specimens.

9. Biological Comparison

The biological assay is considered to be the most specific means for the quantitative determination of adrenaline and noradrenaline in urine. The methods based on the fluorescence reaction, on the other hand, have been shown to be handicapped to a certain degree by unknown non-specific fluorescent substances. Their action was partly eliminated by the above described adjustments in the method. In order to find out the influence of the interfering substances the urinary adrenaline and noradrenaline were estimated simultaneously by both biological and fluorimetric methods.

Urine specimens were collected from two patients who had undergone an extensive surgical operation, as in these conditions an elevated excretion of adrenaline and noradrenaline is known to occur. For practical reasons the urine samples could not be extracted and analysed fluorimetrically until 2—4 weeks after

the collection. In the meantime the samples were stored at pH 3—4, most of the time at +2°C.

The extraction for the biological assays was carried out by aluminium oxide adsorption followed by elution with dilute sulfuric acid (PEKKARINEN, to be published). The pH of the eluate was increased to 4 with calcium bicarbonate. The eluates were tested on cat's blood pressure and after the neutralisation with sodium bicarbonate on hen's rectal coecum.[1])

During the lengthy storage there was a tendency for the formation of a strong non-specific fluorescence in the urine samples. This fluorescence, however, could be distinguished from

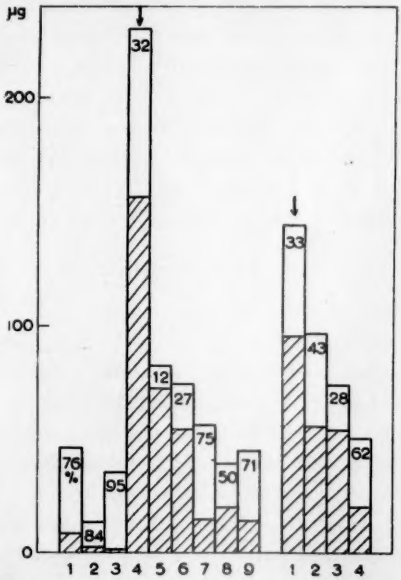

Figure 15. Simultaneous fluorimetric and biological determinations in 13 urine specimens. Ordinate: total adrenaline and noradrenaline excretion in 24 hours. Total column: fluorimetric value. Shaded portion: biological value. Time of surgical operation indicated by arrows. Numbers in the columns indicate the difference in per cents.

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[1]) The biological determinations were performed by Professor A. PEKKARINEN, Department of Pharmacology, University of Turku, which is gratefully acknowledged.

the adrenaline and noradrenaline fluorescence by means of the specificity test. As a consequence, the reading in the blank was greatly increased and an accurate estimation was not possible in many of the samples. In the samples shown in figure 15 this non-specific fluorescence was slight. The time of the operation is indicated by arrows.

In these thirteen comparative analyses the values for the total catechol amine content obtained with both of these methods were definitely correlated. In samples where the biological test (dark columns) gave low values, the fluorescence values also were reduced, and vice versa. In every sample, however, the value obtained by the fluorescence method was markedly higher than in the biological method. The difference in absolute terms between the two values was definite in samples with a high as well as in those with a low adrenaline and noradrenaline content. The difference varied considerably in the different samples. The difference expressed as percentages of the total catechol amine content also varied within wide limits (range 12—95 per cent), being more marked in samples with a low catechol amine content.

Discussion

The fluorescence reaction of adrenaline and noradrenaline is simple and sensitive and offers, therefore, a good opportunity for the development of a chemical method for their determination. Attempts to apply this reaction to quantitative studies of urine have been only partially successful, however, chiefly because of various other fluorescent substances that seriously interfere in the specific evaluation of adrenaline and noradrenaline especially in normal urine. The present investigations and the consequent methodical adjustments have been carried out to eliminate these handicaps.

The inhibition of the fluorescence reaction of adrenaline and noradrenaline and the precipitation in the urine eluates have been almost totally eliminated by using high dilutions of the eluate. Consequently the share of the non-specific fluorescence in the total fluorescence has become proportionally smaller. The oxidation of adrenaline and noradrenaline is also greatly facilita-

ted in high dilutions of the eluate, as complete oxidation is now achieved with only a single treatment using the standard amount of manganese dioxide. This greatly simplifies the carrying out of the procedure. A strong fluorescence of adrenaline and noradrenaline has always been obtained, though it has not been of equal intensity in the different samples. The fluorescence of the sample, therefore, must be compared with the fluorescence of the adrenaline and noradrenaline standards added to every sample.

The use of a high dilution of eluate has brought about a marked disadvantage. The concentration of catechol amines in the final aliquot necessarily is rather low, since it becomes reduced to approximately one-sixth of that in the urine specimen. Consequently the impurities in the distilled water and the laboratory equipment are able to interfere considerably. This can be eliminated only by using the utmost care in this respect. Only vessels washed with distilled water should be used. The determination of these low adrenaline and noradrenaline fluorescences, on the other hand, is easy with a sensitive fluorimeter. The reading for the blank, as compared to the sample reading, is high due to the high dilution of the eluate and causes inaccuracy in the determinations.

The attempts made in these studies to find an accurate method for a differentiation between adrenaline and noradrenaline have not been totally successful. The only procedure found suitable for the differentiation under these conditions is based on the different rate of destruction of adreno- and noradrenolutine in alkaline solutions in the absence of stabilising substances. In the blanks prepared from the urine specimens the fluorescence of adrenaline and noradrenaline disappears in four minutes. These conditions are, therefore, to be considered more favourable for a differentiation of this kind than those found in blood in the studies of LUND (1950) and in urine by GOLDENBERG *et al.* (1954), in both of which the noradrenaline fluorescence disappeared only after 30 minutes. For an accurate differentiation in the manner suggested in the present studies the difference in the rate of destruction of adreno- and noradrenolutine has to

be large. It would be an ideal condition if the destruction of the adrenaline fluorescence would be rapid enough to be total before any decrease in the noradrenaline fluorescence can be detected. This, however, does not occur since the fluorescence of both of these substances fades simultaneously. Therefore the only means to differentiate is to compare the changes in the blank with the changes in the aliquots containing the standard additions of adrenaline and noradrenaline after alkalinisation without ascorbic acid. It is advisable to take the reading when the adrenaline fluorescence has totally faded.

The extraction of the eluate with butyl alcohol is an important step in the specificity of the method. Since a part of adrenaline and noradrenaline is dissolved in the butyl alcohol layer and removed with it, recovery is somewhat lower than in the earlier modification of the method (PEKKARINEN and PITKÄNEN 1955). Extraction is indicated, however, as most of the non-specific fluorescent substances are eliminated thereby. Treatment with formaldehyde, on the other hand, has not been taken into routine use in spite of the promising results obtained with it, because of the possibility that such a highly reactive compound may form fluorescent substances in the blank. This would result in erroneous values for the adrenaline and noradrenaline content.

It is difficult to evaluate the specificity of the present fluorescence method. It was observed in the experiments (figure 5) that in the blank a decrease occurs in the intensity of the fluorescence though the fluorescence due to adrenaline and noradrenaline has totally faded. This indicates that a slight non-specific fluorescence is still present in the readings. The studies concerning the optimal pH of the eluate (figures 6, 9, 10) also suggest the same. The quantitative importance of this non-specific fluorescence cannot be estimated with certainty. The rate of decrease after 4 minutes of alkalinisation of the blank suggests that its share in the total fluorescence is not more than 30 per cent in samples with a physiological adrenaline and noradrenaline concentration.

The concentration of urinary hydroxytyramine is five times greater than the noradrenaline concentration as shown by v.

EULER *et al.* (1951) semiquantitatively on paper chromatograms. Hydroxytyramine obviously yields a fluorescence reaction under the present conditions and consequently interferes with the estimation of adrenaline and noradrenaline. The intensity of the fluorescence reaction of hydroxytyramine is at the most a few per cent of the noradrenaline fluorescence. The share of this substance in the total fluorescence, therefore, remains obviously below 20 per cent.

The simultaneous biological and fluorimetrical determinations were made in urine samples kept for 2—4 weeks at an acid pH before the estimations. Under such conditions adrenaline and noradrenaline may lose biological activity to a slight extent due to racemisation. The values obtained with biological and fluorimetrical methods were therefore comparable only with a certain degree of reservation. The values obtained with the fluorimetrical method were remarkably higher. Whether this was due only to racemisation cannot be stated. The difference was almost as wide in samples with a high as in those with a low adrenaline and noradrenaline content. This suggests that the difference is due to non-specific substances.

It is obvious, therefore, that non-specific substances interfere in the fluorimetric determinations of urinary adrenaline and noradrenaline. The interference is naturally more pronounced in samples with a low adrenaline and noradrenaline content. In the samples with a physiological concentration of adrenaline and noradrenaline their share, as suggested by the biological comparison, is 12—95 per cent of the fluorescence measured. The fluorimetrical method therefore, has only a limited value in the estimation of adrenaline and noradrenaline in physiological concentrations. The specificity of the method naturally increases proportionally to the catechol amine concentration. In the concentrations that are found, for example, in cases of pheochromocytoma the fluorescence due to non-specific substances is only a few per cent of the total.

The adsorption technique and the procedure for development of the fluorescence in the fluorimetrical methods of LUND (1952), PEKKARINEN and PITKÄNEN (1955 a, b) and v. EULER and

FLODING (1955) do not differ in principle from those in the present method. It is obvious, therefore, that the non-specific fluorescence interferes also in those methods. An accurate differentiation and specific values can be obtained by chemical means only after total purification and separation of the urinary catechol amines by paper chromatography, as proposed by HAMBERG and v. EULER (1950), GOLDENBERG *et al.* (1954) and GOLDSTEIN and ABELIN (1956). Such a procedure, however, is much too cumbersome for routine use and the recovery usually tends to be low. In the studies where the adrenaline and noradrenaline content is within physiological range a specific determination can be accomplished by biological assay. For the samples with an elevated adrenaline and noradrenaline content, on the other hand, the methods based on the fluorescence reaction are often useful, especially since they are cheaper and less tedious.

STUDIES ON THE INSULIN-INDUCED URINARY OUTPUT OF ADRENALINE IN RATS

Earlier Investigations

The hypoglycaemia induced by insulin is known to result in an increased secretion by the suprarenal medulla (CANNON *et al.* 1924). This release of catechol amines has been shown to occur in the rabbit (WEST 1951, UDENFRIEND *et al.* 1953), and the rat (BURN *et al.* 1950, HÖKFELT 1951, OUTSCHOORN 1951, 1952, ERÄNKÖ 1952, 1955) in studies of changes in the adrenaline content of the suprarenals. A decrease has invariably been seen in the adrenaline content. An increase in the adrenaline concentration in peripheral blood or in blood samples obtained from the suprarenal vein during insulin hypoglycaemia has almost invariably been noticed by several authors, *e.g.*, in the dog by SATO *et al.* (1933), SUZUKI and SAITO (1951), HOLZBAUER and VOGT (1954 a, b), in the cat by DUNÉR (1954), and in man by HOLZBAUER and VOGT (1954 a, b). On the other hand, there are very few reports on the urinary excretion of adrenaline during insulin-induced hypoglycaemia. It was demonstrated by v. EULER and LUFT (1952) that in man the administration of insulin resulted in a ten-fold increase in the excretion of adrenaline. No increase was found in acromegalic patients.[1]) The hypoglycaemia caused by work was also associated with an elevated excretion of adrenaline (v. EULER and HELLNER 1952).

The present experiments were conducted to obtain further information on the insulin-induced adrenaline and noradrenaline excretion under various circumstances.

Only a few reports have been published concerning the urinary excretion of catechols in experimental animals. Using a rather insensitive biological method, FISCHER and LECOMTE (1950 a, b) found no basal

[1]) A similar increase in the adrenaline excretion after insulin administration in man has recently been reported also by ELMADJIAN, F., LAMSON, E. T., FREEMAN, H., NERI, R. & VARJABEDIAN, L.: (J. Clin. Endocr. 1956, *16*, 876).

excretion in the urine of the rabbit but observed biological activity after the intravenous injection of adrenaline and noradrenaline. Similarly, adrenaline was found after its intravenous administration in the urine of the dog (BACQ *et al.* 1951). No spontaneous excretion of adrenaline was found in the dog by BEYER and SHAPIRO (1945) but about 20 per cent of perorally fed adrenaline was detected in the urine. SCHMITERLÖW (1951), using the biological method of v. EULER and HELLNER (1951), found noradrenaline in the urine of rabbits after administration of 3,4-dioxyphenylserine. No data, however, were given concerning the basal excretion. The urine of the dog, rabbit and guinea-pig was shown by HOLTZ *et al.* (1950) to contain sympathomimetic substances in concentrations that are slightly greater than those in human urine. Noradrenaline was shown to be the most important component. Cow urine contains 0.5—1.5 μg. of noradrenaline per 100 cc. (v. EULER and HELLNER 1951).

SCHAYER (1951 a, b) found labelled adrenaline in the urine of rats after the administration of beta-C^{14}-dl-adrenaline subcutaneously or intravenously. A small proportion of the substance was in a conjugated form. After oral feeding the adrenaline was found to be largely in the conjugated form. v. EULER and HEVESY (1953) found biologically active catechol amines in the normal urine of the rat. The excretion was at its lowest during the night.

Material and Methods

Both sexes of an inbred stock of albino rats of Wistar strain were used in these experiments. The rats were divided into several groups according to age and sex. The weight of the animals in each group did not vary more than $\pm$ 25 gm. Several experiments were carried out with each group, and the time allowed for recovery between the experiments was ten days or more, usually two to three weeks. The rats were fed a standard food mixture containing about 60 per cent of carbohydrates.

In order to obtain sufficient quantities of urine and to minimise the error due to irregular emptying of the bladder the urine flow was increased by giving to each rat 5 cc. of tepid water by stomach tube at the beginning of the collection of urine. During the collection the rats were kept in metabolism cages with a loose floor of galvanised wire netting through which all the excretion products were allowed to drop. The cages were mounted on plates covered with a close-meshed wire net to filter the

solids. As a preservative 1 cc. of 2-N acetate buffer solution of pH 3.5 was used for the total amount of urine excreted during a 5-hour period. At the end of the collection the bladder was compelled to empty by gentle pressure. Unless stated otherwise the urine was collected during 5 hours following the administration of insulin and the urine of three rats kept in the same cage was used for one analysis.

Insulin (Novo Terapeutisk Laboratorium, Copenhagen), regular, was administered subcutaneously mostly after an overnight fast. Hypoglycaemic signs set in about 1 hour after the administration in most experiments. The insulin sensitivity varied greatly among the groups of rats but the sensitivity of the rats in one and the same group was almost uniform. The young female rats were most sensitive, the minimal lethal dose for them being approximately 0.1 I.U. of insulin per 100 gm. body weight. The sensitivity was notably higher in summer than in winter. In addition, the sensitivity seemed to vary somewhat at indefinite intervals (a few weeks) without any detectable cause.

The blood sugar was estimated according to the method of HAGEDORN and JENSEN in samples of 0.1 cc. of blood. The samples were taken from the cut end of the rat's tail.

The same difficulties that were stated in the earlier chapters to handicap the evaluation of the adrenaline and noradrenaline content of human urine were also encountered in rat urine in these studies. Especially the precipitation of the salts in the eluate and the inhibition of the fluorescence were disturbing. In view of the high adrenaline concentration of the rat urine due to hypoglycaemia these interfering factors could be totally eliminated by sufficient dilution of the eluate. A dilution of the urine specimen excreted by three rats in 5 hours to 50 cc. and elution with 5 cc. of 1-N sulfuric acid, followed by a 15-fold dilution of the eluate as described on page 37 was found to be the optimal adjustment to overcome these difficulties and to obtain an adequate final concentration of catechol amines. The conditions for the oxidation of adrenaline and noradrenaline, for the development of the fluorescence reaction and for the

differentiation of adrenaline and noradrenaline as presented on pages 37 and 38 proved to be optimal also for these samples. As the number of rats and the collection time were similar in most experiments the fluorescence reaction showed a uniform pattern in the different samples.

The fluorescence reaction of the control samples obtained from hydrated animals receiving no insulin behaved in the specificity tests similarly to the fluorescence found in the samples of human urine described in earlier chapters. It also could be differentiated with the aid of the two blank reactions. The excretion values obtained from 33 control experiments are shown in table 5.

Table 5. Basal excretion. Value (μg. of adrenaline and noradrenaline per rat in five hours) and adrenaline percentage in 33 control experiments.

μg.	%	μg.	%	μg.	%
0.24	64	0.43	44	0.59	33
0.22	59	0.32	54	0.60	27
0.24	57	0.31	38	0.11	86
0.29	72	0.13	20	0.18	69
0.49	44	0.64	51	0.11	50
0.42	32	0.59	57	0.21	43
0.48	37	0.45	38	0.27	48
0.31	38	0.54	51	0.16	57
0.43	44	0.58	63	0.29	41
0.32	54	0.31	41	0.31	38
0.31	38	0.54	22	0.21	57
				Mean=0.36	47

The administration of insulin resulted in a great increase in the intensity of the fluorescence reaction, which in colour and in behaviour in various specificity tests was similar to that produced by adrenaline and noradrenaline. In the differentiation test it behaved typically like that of adrenaline. No increase in the noradrenaline excretion could be observed in any of these experiments.

Biological assays on cat's blood pressure and on hen's rectal coecum and fluorimetric evaluation were conducted with one control sample and one sample collected after insulin administration

Table 6. Biological control. μg. of adrenaline and noradrenaline excreted in five hours per rat. Dose of insulin = 0.8 unit per rat. Average weight 330 gm.

	Basal excretion		Excretion after insulin administration	
	Biol.	Fluor.	Biol.	Fluor.
Noradrenaline	0.26	0.42	0.20	—
Adrenaline	0.16	0.10	3.70	3.80
Total	0.42	0.52	3.90	3.80

(table 6). The values obtained for the basal excretion by both methods were of the same magnitude. The results showed further that the adrenaline excreted after insulin administration and estimated by the fluorescence method was also biologically active. No increase in noradrenaline excretion was detected even in biological testing. It must, however, be kept in mind that in the presence of a surplus (95 %) of adrenaline also the biological testing of the small amounts of noradrenaline is inaccurate.[1])

No attempts were made after an acid hydrolysis to study the portion of adrenaline conjugated in the rat organism.

For the determination of the adrenaline and noradrenaline content of the suprarenals the animals were killed with a blow on the head and the suprarenals were removed immediately and ground in a mortar with two drops of 1-N sulfuric acid. The ground tissue was shaken with 1-N sulfuric acid (4 cc. for a pair of suprarenals). Then 1 cc. of this acid eluate was extracted with 1 cc. of n-butyl alcohol and diluted to 250 volumes. The estimations were carried out by means of the fluorescence reaction along the lines described on page 38.

Results

1. Adrenaline Excretion and Course of Hypoglycaemia

A series of experiments were conducted to investigate the relationship between the course of the hypoglycaemia and the

[1]) The biological estimations were performed by Professor A. PEKKARINEN, Department of Pharmacology, University of Turku, which is gratefully acknowledged.

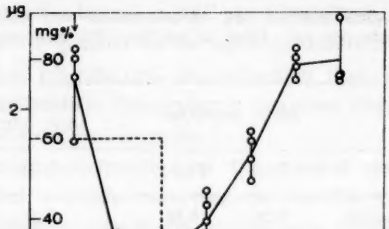

Figure 16. Adrenaline excretion and course of hypoglycaemia. 4 male rats, av. weight 220 gm., injected with 0.12 unit of insulin. Dotted line: amount of adrenaline excreted (µg. per rat during 2 hours). Solid line: mean blood sugar level (mg. per 100 cc.). Arrow: time of insulin injection.

rate of adrenaline excretion. Male rats about six months of age which had fasted overnight were used in these studies. The rats were given various doses of insulin as a single injection. The urine samples were collected at intervals of two hours and the urine flow was kept high with the administration of tap water, 3 cc. per rat at two-hour intervals. Blood sugar samples were taken at one-hour intervals.

In figure 16 are shown the results of an experiment where the test animals (four rats) were given insulin in a dose that was approximately 50 per cent of the lethal dose. The results showed that the highest excretion of adrenaline occurred during the first two hours after the injection of insulin. This high excretion coincided with the decline in the blood sugar level. During the second period of two hours the excretion was remarkably lower, though it still exceeded the basal excretion. Simultaneously the blood sugar content reached its lowest point and began to rise. No increased excretion of adrenaline could be observed during the third two-hour period, while the restoration of the blood sugar level was still in progress.

The results of the various experiments were similar. The highest adrenaline excretion coincided with the decline of the blood sugar level, which occurred within the first two hours after the injection of insulin. This was not dependent on the dose of insulin or the duration of the hypoglycaemia. During the third and fourth hours a sharp decline in the excretion was observed without exception. After this period a slightly increased adrenaline excretion could be detected in a few experiments only. This was possibly due to a retention of some urine in the bladder. The restoration of the blood sugar level often took place during a time when the adrenaline excretion already had declined, in few experiments after the basal excretion level was reached (figure 16). This decline of the excretion before a complete restoration of the blood sugar level occurred was observed also in experiments where relatively small doses of insulin were administered. It therefore could not be due to a depletion of the adrenal medullary stores.

2. Effect of Various Doses of Insulin

In the experiment shown in figure 17 a group of rats was divided into thirteen subgroups with three rats in each. The

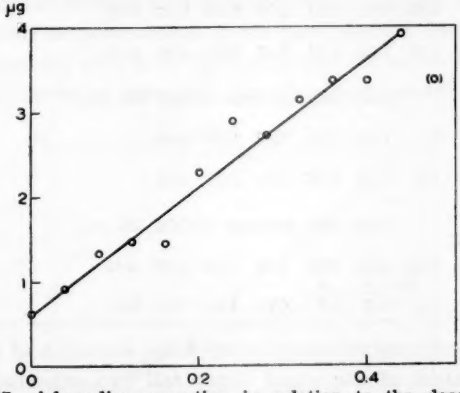

Figure 17. Adrenaline excretion in relation to the dose of insulin. 39 female rats, av. weight 310 gm. In the group in parentheses one rat died with convulsions during the collection period. Ordinate: μg. of adrenaline per rat excreted in 5 hours. Abscissa: units of insulin injected per rat.

subgroups were given increasing doses of insulin after overnight fasting. The adrenaline contents were determined in urine samples collected during the five hours following the injection.

The excretion of adrenaline (figure 17) increased as larger doses of insulin were administered. The increase was linear in proportion to the insulin dosage within a wide range, extending from small doses that exerted no overt effect on the test animal to doses sufficient to produce severe coma with convulsions. The linearity was broken only when collapse or death of the test animal occurred during the time of the collection.

The results of four other experiments were similar to those shown in figure 17 (table 7).

Table 7. Effect of various doses of insulin on adrenaline excretion (μg. in five hours per rat).
+One or several of the rats died during the collection period.

	Male rats, average weight 300 gm.										
Insulin units per rat	0	0.08	0.12	0.16	0.20	0.28	0.32	0.36	4.40	0.44	0.52
Adrenaline excretion	0.8	1.5	1.9	2.5	2.7	3.3	4.4	3.6	3.9	4.8	5.6
	Female rats, average weight 280 gm.										
Insulin units per rat	0.04	0.08	0.12	0.16	0.20	0.24	0.28				
Adrenaline excretion	1.10	1.60	2.10	2.80	2.80	4.30	3.70+				
	Male rats, average weight 300 gm.										
Insulin units per rat	0	0.12	0.24	0.36	0.48	0.60					
Adrenaline excretion	1.0	1.20	2.20	2.90	3.30	3.80					
	Male rats, average weight 330 gm.										
Insulin units per rat	0.40	0.60	0.80	1.20	1.60	2.00	2.40				
Adrenaline excretion	3.6	4.7	6.6	4.7+	4.3+	4.6+	5.4+				

To study the relationship between the excretion of adrenaline and the extent of the blood sugar fall two experiments were conducted where the course of the hypoglycaemia was followed together with the adrenaline excretion in rats receiving various doses of insulin.

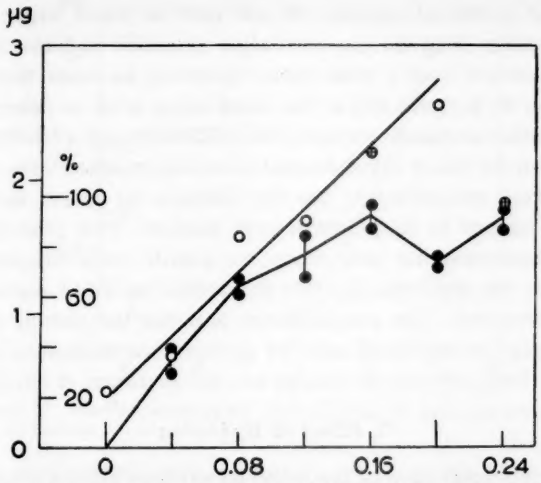

Figure 18. Adrenaline excretion in relation to blood sugar fall induced by various doses of insulin. 21 female rats, av. weight 220 gm. Abscissa: units of insulin injected per rat. Blood sugar fall expressed in per cents of the pre-injection level, adrenaline excretion in μg. per rat in 5 hours. O=excretion of adrenaline. +=excretion of adrenaline in group in which 1 rat died during collection period. ●=blood sugar fall.

The maximum falls of the blood sugar (figures 18 and 19) are expressed as percentages of the pre-injection levels. As can be seen from the results in figures 18 and 19, an increase in the adrenaline excretion occurred already after insulin doses that

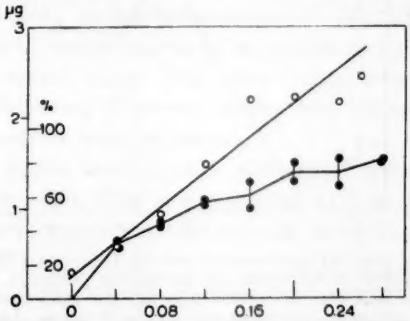

Figure 19. Similar experiment as in figure 18. Male rats, av. weight 250 gm.

produced a fall of approx. 30 per cent in blood sugar. The stoichiometry between the adrenaline excretion and the insulin dosage existed over a wide range extending to doses that produced an 80 per cent fall in the blood sugar level as determined by the rather unspecific method of HAGEDORN and JENSEN. The decline in the blood sugar concentration was marked when small doses were administered; the fall induced by larger doses of insulin seemed to be proportionally smaller. This phenomenon was conspicuous not only when the results were computed in per cents but when the absolute declines in the blood sugar level were compared. The stoichiometry between the insulin dosage and the fall of the blood sugar level, therefore, seemed to be less distinct than between the dosage and the excretion of adrenaline.

3. Effect of Hydration

The administration of tap water by stomach tube in a quantity of approximately 2.5 per cent of the body weight as used in most of these experiments necessarily alters the metabolic state of the rats. At least it must be considered to be a marked stress

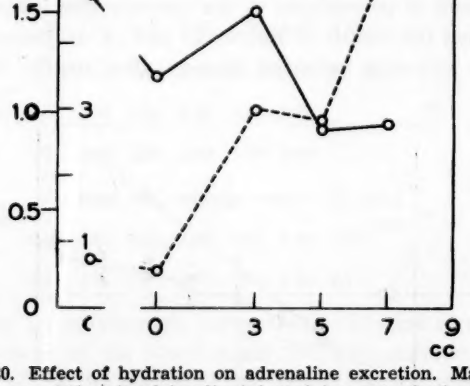

Figure 20. Effect of hydration on adrenaline excretion. Male rats. av. weight 240 gm., 0.12 unit of insulin injected per rat. Ordinate: amounts of adrenaline and urine excreted per rat in 5 hours. Abscissa: dose of water administered per rat. Solid line: adrenaline excretion. Dotted line: rate of urine flow.

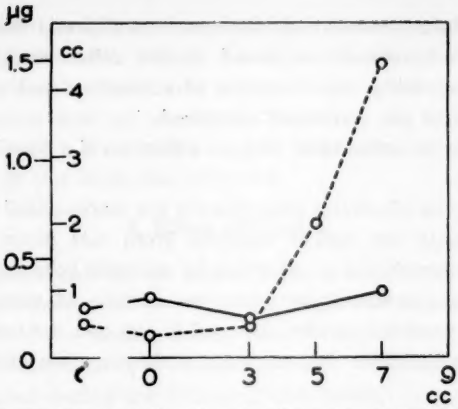

Figure 21. Similar experiment as in figure 20, but without administration of insulin.

on the test animal, with a possible effect on the excretion of adrenaline. It was observed in the course of these investigations that a high excretion of adrenaline elicited by insulin constantly was associated with a considerably increased diuresis. Closer study of this question seemed therefore indicated.

The rats in three experiments (the results of one of which are shown in figure 20) were given various amounts of tepid water (0—7 cc. per rat) by stomach tube at the time of insulin administration. In addition, three rats (group C) had no access to water for 24 hours before the insulin injection and no water was given to them during the experiment. The rats were given an equal dose of insulin known to be capable of inducing moderate hypoglycaemic signs. The urine samples were collected during the following five-hour period and their volumes and adrenaline contents were estimated.

The rats loaded most heavily with water showed the least hypoglycaemic signs. The administration of 7 cc. of water induced an approximately ten-fold increase in the diuresis (figures 20 and 21) as compared to the urine flow of rats that were not water-loaded. The excretion of adrenaline, nevertheless, seemed to be independent of the rate of urine flow even over this wide range.

No definite correlation between the diuresis and the excretion of adrenaline was found in two other similar experiments. If anything, the excretion of adrenaline had a tendency to decrease as the waterload increased.

The rate of urine flow had no effect on the basal excretion (figure 21).

The results of similar experiments are summarised in table 8, in which only the results obtained from rats given less than 3 cc. and more than 6 cc. of water by stomach tube are included. No positive correlation between the volume of urine and the adrenaline excreted can be observed in one and the same experiment. This indicates that no large scale retention of adrenaline

Table 8. Effect of hydration. Adrenaline (μg. per rat) and urine volume (cc. per rat) excreted in five hours after insulin injection. M=mean.
+One or several of the rats died during the collection period.

	< 3 cc.		> 6 cc.	
	μg.	cc.	μg.	cc.
Experiment 1.	3.12	4	1.85	8
Female rats, average weight 300 gm., injected with 0.48 unit of insulin per rat.	2.40	4	1.75	8
	2.75	6	2.65	9
	1.98	6	2.65	13
			2.50	10
	M=2.56		M=2.28	
Experiment 2.	1.58	1	0.92	3
Male rats, average weight 210 gm., injected each with 0.12 unit of insulin.	1.16	1	0.94	6
	1.51	3	0.92	4
	1.10	1	0.75	5
	0.82	1		
	0.70	4		
	M=1.14		M=0.88	
Experiment 3.	2.45	1	2.05	6
Male rats, average weight 300 gm., injected each with 0.20 unit of insulin.	2.50	3	1.70	9
	M=2.48		M=1.88	
Experiment 4.	2.95	7	3.08	15
Female rats, average weight 250 gm., injected each with 0.32 unit of insulin.	3.29+	6	3.82	17
	3.21	4	3.42+	18
	3.42	10	3.02	16
	4.38	7	2.42+	13
	3.15	7	3.10	13
	M=3.39		M=3.11	

in the bladder occurred when the urine was collected during a five-hour period. The higher excretion of adrenaline in rats receiving a smaller water load was striking in experiments 1—3. The difference was not significant, however. In experiment 4, where the rats were given a nearly lethal dose of insulin, no difference of this kind was observed.

4. Effect of Fasting

The test animals in the experiments presented in figures 22 and 23 were fasted for 0—70 hours, with free access to water. At the end of the fasting period equal doses of insulin and tap water were administered simultaneously. The urine samples were collected during the following five hours.

After a fast of only a few hours the insulin-induced excretion was low. The excretion rose rapidly as the fasting time was prolonged. The highest values were obtained during the remainder of the first 24 hours. After a longer fasting time the insulin-induced excretion of adrenaline decreased steadily. The fast of 70 hours resulted in an adrenaline excretion that was

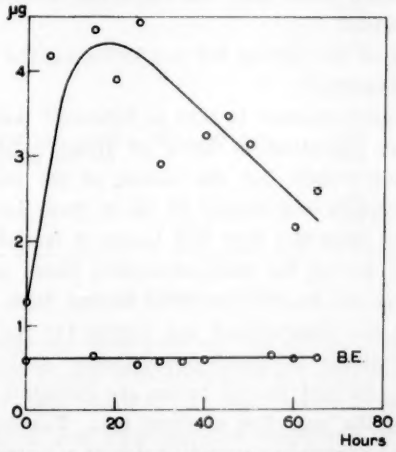

Figure 22. Effect of starvation on insulin-induced adrenaline excretion and on basal excretion. Female rats, av. weight 210 gm., injected each with 0.2 unit of insulin. B.E.=basal excretion. Ordinate: adrenaline excreted per rat in 5 hours. Abscissa: fast in hours.

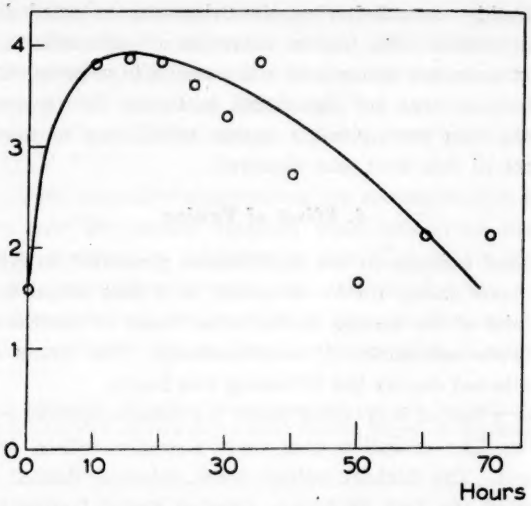

Figure 23. Similar experiment as in figure 22. 33 female rats, average weight 250 gm, injected with 0.3 unit of insulin per rat.

about 50 per cent lower than the excretion induced during the postprandial period.

The fasting in this extent did not influence the rate of basal excretion (figure 22).

Six experiments similar to that in figure 22 were conducted. The adrenaline excretion in some of these experiments was scattered rather widely but the course of the insulin-induced adrenaline excretion was similar in all of them, i.e., a rapid increase occurred after the first few hours of fast, followed by a high excretion during the post-adsorptive phase and a gradual decrease during the second and third fasting days.

In table 9 are summarised the values for the excretion of adrenaline obtained in these experiments. Only the fasting periods of 10—20 and 60—70 hours are included. Each figure is the mean of the excretion of three rats. The decrease in the insulin-induced adrenaline excretion due to a longer fasting time is conspicuous in all the experiments quite independently of the dose of insulin administered and of the group of rats used.

Table 9. Effect of starvation. Adrenaline (μg.) excreted in 5 hours per rat after injection of insulin. M=mean.

	Fasting time	
	10—20 hours	60—70 hours
Male rats, average weight 300 gm., 0.4 unit of insulin per rat.	4.75 4.20 M=4.48	2.55 3.20 M=2.88
Female rats, average weight 200—300 gm., 0.12 unit of insulin per rat.	2.25 3.30 M=2.78	1.95 1.40 M=1.68
Female rats, average weight 200—300 gm., 0.3 unit of insulin per rat.	3.80 3.80 M=3.80	2.30 M=2.30
Male rats, average weight 200—300 gm., 0.2 unit of insulin per rat.	0.90 0.95 0.80 M=0.88	0.70 M=0.70
Female rats, average weight 200—300 gm., 0.1 unit of insulin per rat.	1.05 1.76 2.06 2.13 2.16 2.60 M=1.96	0.85 1.05 1.14 1.20 1.22 1.63 1.74 M=1.26
Female rats, average weight 200—300 gm., 0.1 unit of insulin per rat.	1.41 1.63 1.74 1.90 1.63 M=1.66	1.19 1.36 1.40 1.50 1.61 M=1.41

5. Effect of Diet

The test animals were kept on two different diets. The one contained about 85 per cent starch, 10 per cent casein and 5 per cent bran, the other about 85 per cent casein, 10 per cent starch, 5 per cent bran, and hydrogenated vegetable oil ad lib. The rats were kept on these diets for 3.5—6 days and then fasted overnight. The five-hour excretion of adrenaline after the administration of an equal dose of insulin to each rat was deter-

mined. The results of the three experiments are summarised in table 10.

Table 10. Effect of diet. Adrenaline excretion (μg. in five hours per rat) after insulin administration. M=mean.
+One of the three rats succumbed during the collection period.
++Two —„— —„— —„— —„—

	Diet rich in	
	Sugar	Protein and Fat
Experiment 1.		
Female rats, average weight 210 gm., 0.1 unit of insulin per rat.	4.20+	2.65
	2.40++	3.00
	2.05+	3.35
	1.55	2.50
	2.15+	2.50
	1.80	2.20
		1.80
	M=2.02	M=2.57
Experiment 2.	1.80	1.20
Female rats, average weight 210 gm., 0.05 unit of insulin per rat.	2.10	1.10
	1.80+	1.20
	2.27	1.35
	M=1.99	M=1.21
Experiment 3.	2.20+	0.30
Male rats, average weight 280 gm., 0.15 unit of insulin per rat.	1.40	0.43
	2.80	0.52
	2.90	0.39
	2.80	0.37
	3.70	0.47
	M=2.63	M=0.41

The rats fed a carbohydrate-rich diet showed definitely stronger hypoglycaemic signs than their counterparts. In experiment 1, five out of eighteen carbohydrate-fed rats died with convulsions while the protein-fed but otherwise identical rats showed only slight hypoglycaemic signs. Owing to the high mortality in this group the values for the excretion of adrenaline are not reliable. The same group of rats was used in experiment 2, in which they were injected with a much smaller dose of insulin. Again the sugar-fed group exhibited the stronger hypoglycaemic signs. The excretion of adrenaline was definitely lower in the protein-fed rats. A still wider difference in the excretion

is seen in experiment 3. The adrenaline excretion did not notably exceed the basal excretion level in the protein-fed group, whereas it was at least five times as great in their counterparts.

In experiment 3 the difference in the insulin-induced excretion of adrenaline between the sugar-fed and protein/fat-fed rats was much wider than in experiment 2. The results of the following experiment, in which the effect of the diet and fasting were studied simultaneously, suggest that this was due to difference in the fasting times (10 hours in exp. 3, 21 hours in exp. 2). The rats in the experiment (figure 24) were kept on the carbohydrate-rich and on the carbohydrate-poor diets for 6—7 days and fasted for various times (8—30 hours). The adrenaline excretion was then determined after the administration of insulin in equal doses. The blood sugar level was followed in the rats that were fasted for 10 and 30 hours.

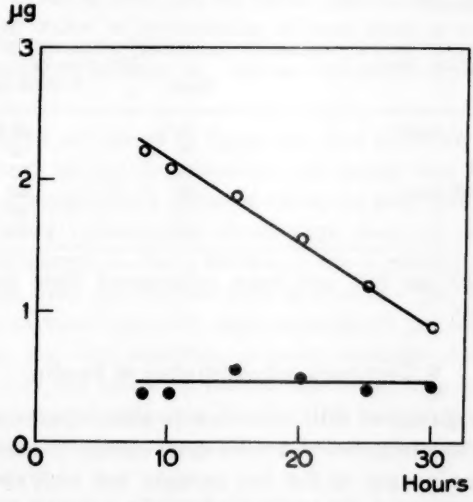

Figure 24. Effect of fasting on insulin-induced adrenaline excretion in rats fed on carbohydrate-rich and carbohydrate-poor diets. Female rats, av. weight 220 gm., dose of insulin=0.06 unit per rat. Ordinate: adrenaline excreted per rat in 5 hours. Abscissa: fast in hours. O=carbohydrate-rich diet. ●=carbohydrate-poor diet.

Insulin induced in the rats that had been fed a carbohydrate-rich diet and fasted for 8—10 hours a pronounced coma with convulsions. Prolongation of the fast resulted in a marked diminution of the hypoglycaemic signs. Injection of the same dose of insulin had no overt effect on the protein/fat-fed rats. The values for the adrenaline excretion are shown in figure 24. Prolongation of the fast resulted in a marked decrease in the excretion of adrenaline in the sugar-fed rats. An equal dose of insulin induced after a fast of 30 hours an adrenaline excretion of only about one-third of that occurring after the 8—10 hours' fast. The excretion in the protein-fed rats was only slightly above the basal excretion level and apparently was not influenced by a fast of this length. The fall in the blood sugar level (table 11) was more pronounced in the sugar-fed group, and in

Table 11. Effect of diet and starvation on blood sugar fall induced by insulin. Blood sugar fall expressed as percentage of the pre-injection level. Female rats, average weight 220 gm. Dose of insulin = 0.06 unit per rat.

Fasting time	Diet rich in	
	Sugar	Protein and Fat
10 hours	70 %	53 %
	70	52
	59	30
30 hours	56	29
	42	39
	56	12

both groups the fall was more pronounced after the shorter fasting time.

6. Continued Administration of Insulin

To six groups of rats, three rats in each, equal doses of insulin were administered that were large enough to induce severe hypoglycaemic signs in the test animals, but with spontaneous recovery. The same dose of insulin was given to each rat every other day, preceded by fasting for ten hours. The excretion of adrenaline was determined in urine samples collected during five hours after the injection.

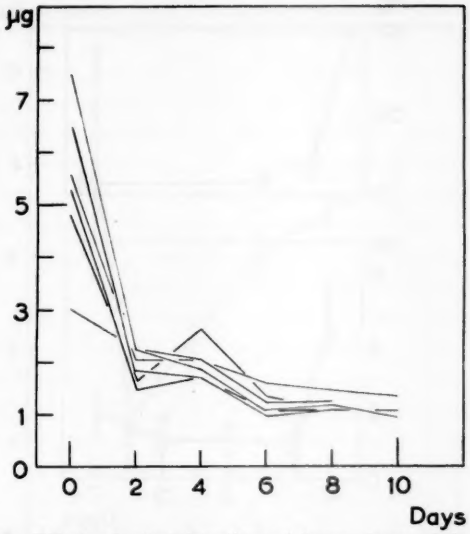

Figure 25. Effect of administration of large doses of insulin every other day on adrenaline excretion. 18 female rats, av. weight 230 gm., dose of insulin=0.1 unit per rat. Ordinate: adrenaline excreted per rat in 5 hours.

The results are shown in figure 25. The excretion of adrenaline induced by the first injection of insulin was 3—7.5 µg. per rat in 5 hours. These values were of the same order as those found in other experiments where large doses of insulin had been administered. A sharp decline in the adrenaline excretion was noticed after the second dose of insulin. The amount of adrenaline excreted was only approximately 25 per cent of that elicited by the first injection. A more moderate decline was observed after the following insulin injections. The amounts of adrenaline excreted after the fourth, fifth and sixth injections were similar and they exceeded somewhat the average basal excretion level.

The first injection of insulin induced the most severe hypoglycaemic signs in the test animals.

In order to observe whether this marked decline in the adrenaline excretion was due to a depletion of the suprarenal

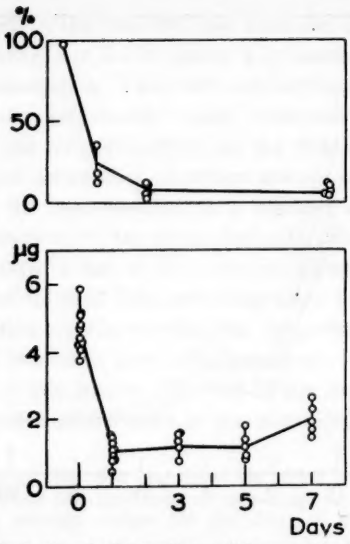

Figure 26. Effect of administration of large doses of insulin every other day on adrenaline excretion and suprarenal catechol amine stores. Male rats, av. weight 330 gm., 0.8 unit of insulin injected per rat. Lower curve, ordinate: adrenaline excreted per rat in 5 hours. Upper curve, ordinate: suprarenal catechol amine content expressed as percents of amount found in intact rats in the same group.

medullary stores, an identical experiment was carried out during which some of the animals were sacrificed and the catechol amine content of the suprarenals was determined.

The prompt decline in the adrenaline excretion was in this experiment as conspicuous as in the former (figure 26). The catechol amine content of the suprarenals showed a parallel course. The first injection of insulin produced a 70 per cent fall in the content, which declined further to only a few per cent of the pre-injection level as the insulin injections were continued.

7. Restoration of Adrenaline Excretion

The excretion of adrenaline elicited by hypoglycaemia can be brought to a low level by repeated injections of insulin, as demonstrated in figure 25. This occurs simultaneously with a

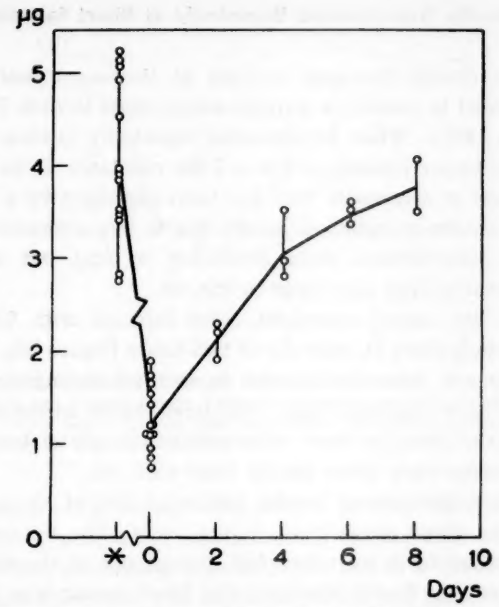

Figure 27. Restoration of insulin-induced adrenaline excretion. 39 female rats, av. weight 250 gm., dose of insulin=0.2 unit per rat. Ordinate: adrenaline excreted per rat in 5 hours.

depletion of the adrenaline stores of the body. In the following experiment (figure 27) the rate of restoration of the adrenaline excretion was studied.

A group of rats were on two successive days given an equal dose of insulin large enough to induce severe signs of hypoglycaemia, with spontaneous recovery. The adrenaline stores were depleted in this way, as indicated by the low excretion elicited by the second administration of insulin (figure 27). Nine rats were then taken from this group every other day and injected under identical conditions with the same dose of insulin.

The insulin-induced adrenaline excretion increased rapidly during the first few days after the depletion of the adrenaline stores. A nearly normal level was reached in four days. Total recovery occurred in about six to eight days.

8. Insulin Administered Repeatedly at Short Intervals

Insulin causes profound changes in the sugar metabolism when allowed to produce a hypoglycaemic state in man (SOMOGYI 1949, 1950). When administered repeatedly to man it loses some of its hypoglycaemic power and the resistance of the organism to sugar is decreased. This has been explained by a mobilisation of insulin-antagonistic agents due to hypoglycaemia (SOMOGYI). Experiments were conducted to find out whether changes of this kind also occur in the rat.

Three rats, fasted overnight, were injected with 0.05 unit of insulin four times at intervals of two hours (figure 28). A total of 0.2 unit was thus administered to each rat during six hours. The urine flow was kept high with tepid water administrations and the urine samples were collected at intervals of two hours. Blood samples were taken hourly from each rat.

The first injection of insulin induced a fall of about 45 per cent in the blood sugar level in one hour. The following injections called forth a further fall, though not of the same extent. After the fourth injection the blood sugar was reduced

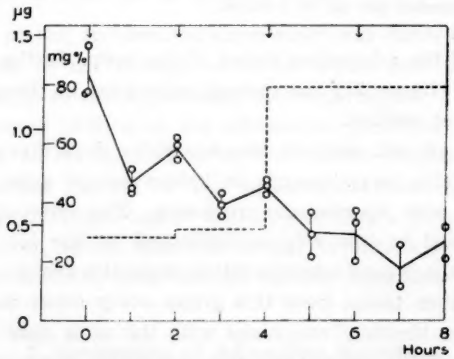

Figure 28. Effect of insulin administered at 2-hour intervals. 3 male rats, av. weight 310 gm., injected with 0.05 unit of insulin per rat at times indicated by arrows (0.2 unit in total). Ordinate: adrenaline excreted per rat in 2 hours and blood sugar in mg. per 100 cc. Solid line: mean of blood sugar level. Dotted line: adrenaline excretion.

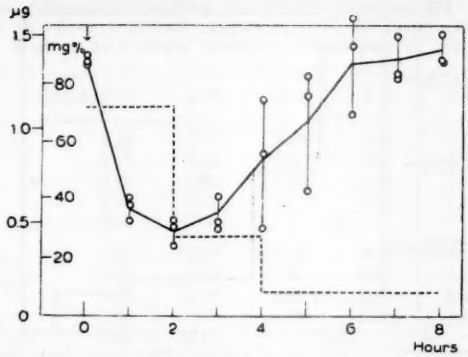

Figure 29. Effect of a single injection of insulin. 3 male rats, av. weight 310 gm., injected with 0.2 unit of insulin per rat. Solid line: mean of blood sugar level. Dotted line: adrenaline excreted per rat in 2 hours. Arrow: time of insulin injection.

to approximately 20 per cent of the pre-injection level. The excretion of adrenaline was increased by the first injection of insulin. The succeeding injections resulted in a definite further rise in the excretion. The condition of the rats grew worse after each injection. After the last injection they were convulsant and one of them died.

For comparison another experiment was conducted simultaneously. Three rats, identical to those used in the former experiment, were given a single injection of 0.2 unit of insulin, i.e., the dose given in the preceding experiment in six hours. The results are shown in figure 29.

The administration of this amount of insulin as a single injection caused a fall of about 65 per cent in the blood sugar content as compared to the final decline of 80 per cent in the preceding experiment. The maximum rate of excretion of adrenaline was as high in both experiments but it was limited in the latter experiment to a shorter period. The administration of the insulin dose during a period of six hours resulted in the excretion of a larger total amount of adrenaline.

The course of the adrenaline excretion in another identical experiment is shown in figure 30.

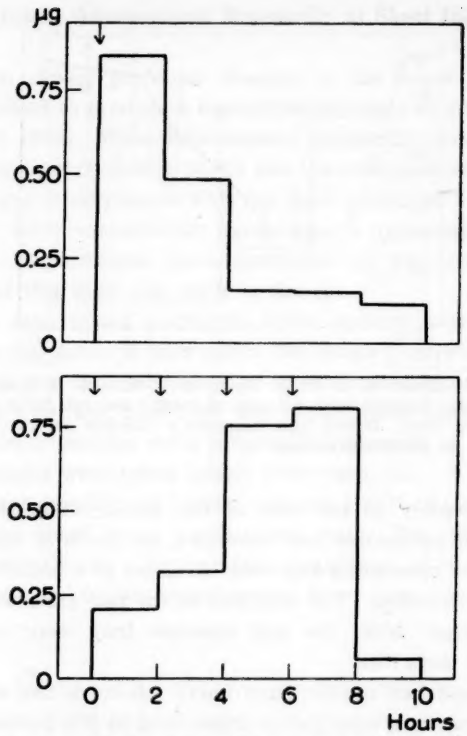

Figure 30. Comparison of effects of a single injection and repeated injections of insulin on adrenaline excretion. Upper figure: 3 female rats, av. weight 220 gm., injected once with 0.16 unit of insulin. Lower figure: similar rats injected 4 times with 0.04 unit of insulin at 2-hour intervals. Ordinate: adrenaline excreted per rat in 2 hours. Arrow: time of insulin injection.

The results of five similar experiments are summarised in table 12. In the lower part of the table are collected the adrenaline excretion values (μg. per rat during two hours) obtained after a single injection of insulin; the upper table gives the values obtained when the same dose of insulin is administered to identical rats in four injections at two hours' intervals. The total adrenaline excretion was higher in every experiment in the latter group.

Table 12. Adrenaline excretion (μg. in two hours per rat) when insulin injected at two-hour intervals (upper part of the table) and when the same dose is given in a single injection (lower part of the table).

	Hours 0—2	2—4	4—6	6—8	8—10	TOTAL
Experiment 1.	0.19	0.36	0.84	0.90	0.02	2.31
—„— 2.	0.18	0.46	0.77	0.80	0.37	2.58
—„— 3.	0.23	0.68	1.60	0.80	0.35	3.66
—„— 4.	0.26	0.45	1.00	0.55	0.26	2.52
—„— 5.	0.37	0.39	1.72		—	2.48
Experiment 1.	0.82	0.57	0.17	0.17	0.06	1.79
—„— 2.	0.88	0.53	0.24	0.31	0.14	2.10
—„— 3.	1.20	0.23	0.26	0.10	0.23	2.02
—„— 4.	1.05	0.62	0.19	0.10	0.26	2.22
—„— 5.	1.00	0.25	0.48		—	1.73

The choice of an adequate dose of insulin for the demonstration of this cumulative action of insulin was difficult. With doses that were too large the test animals tended to succumb already after the second injection. The action of too small doses, on the other hand, was noticed only in rats to which the entire dose was administered as a single injection.

9. Effect of Adrenalectomy

Adrenalectomy was made in one stage by dorsal approach. Six male rats (average weight 330 gm.) and twelve female rats (average weight 300 gm.), all about one year old, were used in these experiments. The adrenalectomised animals were maintained on the standard diet with free access to tap water and 1 per cent saline solution. Desoxycorticosterone acetate, 1 mg. per rat in oily solution, was given every other day and cortisone acetate, as a water suspension in doses shown in table 13, was given daily, both subcutaneously. The rats were maintained on the cortisone dose for at least four days before the experiment. The animals were fasted for fourteen hours before the experiments. The injections of insulin were given two hours after the cortisone administration. Several experiments were carried out with each rat, the time for recovery being at least four days. Between the experiments, five-hour

urine samples were collected without insulin administration for the determination of the basal excretion (table 14).

The adrenalectomised animals were markedly sensitive to insulin, and an amount of 7.5 mg. of cortisone acetate daily was needed to normalise the sensitivity. Moreover, the sensitivity varied considerably in the different rats.

In experiments I, II and V (table 13) the rats showed moderate hypoglycaemic signs. The blood sugar was followed hourly in rats selected at random from these groups. The blood sugar fall was moderate. In experiment VI all the rats showed marked hypoglycaemic signs, most of them collapsed and were given intraperitoneal injections of glucose during the collection of the urine. The blood sugar fall in six rats selected at random from this group was pronounced.

In experiments III, IV, VII and VIII relatively smaller amounts of insulin were administered and only a few animals

Table 13. Insulin-induced adrenaline excretion after adrenalectomy (μg. in five hours per rat).

Experiment	Cortisone acetate, mg. per rat daily	Insulin units per rat	Days after operation	Total catechol excretion μg.	Adrenaline percentage	Blood sugar fall in per cent
MALE RATS						
I	1.5	0.3	7	0.25	36	45, 64
I	1.5	0.3	7	0.24	4	38
II	2.5	0.12	12	0.43	27	33, 0
III	2.5	0.10+q.s.	18	0.17	64	66, 53, 50
IV	7.5	0.30+q.s.	23	0.17	26	77. 70
FEMALE RATS						
V	2.5	0.08	3	0.33	42	20, 37, 32
VI	2.5	0.16	4	0.33	62	58, 53, 49
VI	2.5	0.16	4	0.21	70	39, 61, 67
VI	2.5	0.16	4	0.31	30	
VII	2.5	0.10+q.s.	9	0.23	31	25, 50, 50
VII	2.5	0.10+q.s.	9	0.27	31	
VII	2.5	0.10+q.s.	9	0.92	35	
VIII	7.5	0.30	14	0.23	22	67, 72, 71
VIII	7.5	0.30	14	0.17	33	61
VIII	7.5	0.30	14	0.19	36	
				Mean=0.25	36	

showed definite hypoglycaemic signs. Rats that were in a good condition were therefore given additional insulin injections. Most of the rats had in this way undergone a hypoglycaemic period during the collection of the urine sample. The blood sugar falls in the rats selected at random in these groups were pronounced.

Table 14. Basal excretion after adrenalectomy (μg. in five hours per rat).

Cortisone acetate mg. per rat daily	Days after operation	Total excretion μg.	Adrenaline percentage
MALE RATS			
1.5	4	0.18	6
1.5	4	0.18	42
1.5	10	0.24	20
1.5	10	0.18	31
2.5	16	0.24	26
7.5	21	0.11	16
7.5	26	0.16	27
0	30	0.16	27
FEMALE RATS			
2.5	7	0.27	15
2.5	7	0.35	19
2.5	7	0.18	10
7.5	12	0.19	32
7.5	12	0.39	18
7.5	12	0.22	22
7.5	17	0.28	12
7.5	17	0.29	43
0	22	0.42	37
0	22	0.56	27
		Mean=0.26	23

The mean basal adrenaline and noradrenaline excretion in five hours was 0.26 μg. per rat, with 23 per cent adrenaline (table 14). Insulin hypoglycaemia was not capable of increasing the adrenaline and noradrenaline excretion in adrenalectomised rats in any of these experiments (table 13). This was independent of the dose of insulin and the amount of cortisone on which the rats were maintained, although in all the experiments the animals were in a definite hypoglycaemic state. The mean total adrenaline and noradrenaline excretion during insulin hypoglycaemia was 0.25 μg. per rat in five hours with an average adrenaline percentage of 36 per cent.

Discussion

The adrenaline and noradrenaline content of the urine of the rat can be evaluated with the aid of the fluorescence reaction. The disadvantages of the method that were encountered in the studies on human urine were considerable also in this connection. Most samples in the present study contained a high adrenaline/noradrenaline ratio which made accurate differentiation difficult. The specificity of this fluorescence method evidently was not perfect at low catechol amine concentrations, but owing to the high adrenaline content of the samples after the administration of insulin the values as a whole may be considered reliable.

The values for the basal excretion (table 5) were most liable to have been affected by the error due to non-specific fluorescent substances. The biological control, however, showed that this could not be of great importance even at these low adrenaline and noradrenaline concentrations. The urine samples were collected after a manipulation such as the administration of water in an amount of 2—3 per cent of body weight by a stomach tube. It must therefore be held that the collections were accomplished during a condition of marked stress. How much this may have affected the catechol amine output is not known. However, the results in figures 20 and 21 suggest that it has not been of any great importance. In any case the figures obtained for the basal adrenaline and noradrenaline concentration were of the same order as those obtained by, e.g., HOLTZ *et al.* (1950) for other animal species.

The highest value for the adrenaline excretion was 7.5 μg. per rat during five hours after the administration of insulin. In the experiments in which the rats exhibited distinct hypoglycaemic signs the five-hour excretion of adrenaline varied in most experiments between 2 and 4 μg. per rat, corresponding to an output of approximately 0.9—1.2 μg. per 100 gr. body weight in the same period. The adrenaline excretion thus exceeded the basal adrenaline and noradrenaline excretion level about ten to twenty-fold. A ten-fold increase in the adrenaline

excretion was found by v. EULER and LUFT (1952) after the administration of insulin in a dose that was capable of inducing hypoglycaemic symptoms in man. A ten-fold or larger increase in the secretion of the suprarenal medulla has been observed during insulin hypoglycaemia also in the dog (YEN *et al.* 1933, HOLZBAUER and VOGT 1954 a, b) and in the cat (DUNÉR 1954).

The adrenaline stores of the rat, which almost totally are concentrated in the suprarenals, contain about 15—30 μg. of adrenaline per 100 gr. body weight (HÖKFELT 1951, OUTSCHOORN 1952). This, when compared with the adrenaline output during insulin hypoglycaemia, suggests that almost 10 per cent of the adrenaline stores were excreted in the urine in the free form. In man, on the other hand, v. EULER, LUFT and SUNDIN (1954) have shown that only about 1 per cent of the intravenously administered adrenaline is excreted in the urine. FISCHER and LECOMTE (1950 a) found for the rabbit and BACQ *et al.* (1951) for the dog slightly higher percentages (8.5 and 2—6 per cent, respectively). This proportionally high excretion of adrenaline as compared to the amount available in the body stores can be explained at least in part by an increased synthesis in the suprarenal medulla which is shown to occur in connection with insulin administration (UDENFRIEND *et al.* 1953). The quantitative importance of the increased synthesis during the five hours of collection of the urine sample cannot be stated. The observation of WEST (1951) that during an insulin hypoglycaemia in the rabbit a marked initial increase occurs in the adrenaline content of the suprarenals suggests that the increased synthesis may be of a high order. OUTSCHOORN (1952) similarly found in the rat an initial increase of 30—50 per cent in the noradrenaline content of the suprarenals. A similar initial increase was found also by HÖKFELT (1951) in the rat. It is also possible that due to the high insulin-induced discharge of adrenaline into the general circulation a greater portion of this substance escapes destruction by the tissues. It has been shown (CELANDER and MELLANDER 1955) in the cat that the elimination of adrenaline and noradrenaline from the blood by the tissues is proportionately decreased when doses greater than

physiological are infused. Similarly, greater proportions of noradrenaline are recovered in urine in man when this substance is infused in increasing amounts (ELMADJIAN *et al.* 1956 b).

No increase was found in the excretion of noradrenaline in the present investigation. This is in accordance with the results of v. EULER and LUFT (1952) in man. The selective release of adrenaline with no increase in the noradrenaline secretion during the insulin hypoglycaemia has been an almost uniform finding (HÖKFELT 1951, 1953, DUNÉR 1954, HOLZBAUER and VOGT 1954 a, b). It is remarkable that in the present studies no noradrenaline was excreted even during treatment with successive injections of large doses of insulin, albeit noradrenaline almost totally disappeared from the suprarenals. It is possible, however, that a slight increase in the noradrenaline excretion escaped detection because of inaccuracy in the differentiation in the presence of a surplus of adrenaline. Successive injections are known to deplete the noradrenaline stores in the suprarenals also in the cat (HÖKFELT, cited by DUNÉR 1954).

It has been postulated that the blood sugar level declines proportionately only when small doses of insulin are administered. Due to counter-regulatory mechanisms the rate and extent of the blood sugar fall is proportionately smaller after large doses of insulin in man (SOMOGYI 1949, VOLK and LAZARUS 1953). This has been observed also in the dog but not in the cat (ZUCKER and BERG 1937 a). The results in figures 18 and 19 suggest that this is the case also in the rat. In any case the results show that the stoichiometry between the adrenaline excretion and the dosage of insulin was more striking and within a wider range. In the evaluation of the effect exerted by insulin on the organism, therefore, the estimation of the adrenaline excretion appears to be more appropriate than the determination of the fall of the blood sugar.

During the period of collection the urine samples were exposed to evaporation. Consequently the figures for the urine volumes in table 8 and figures 20 and 21 cannot be considered accurate. The adrenaline content in the most concentrated samples was about 380 μg. per 100 cc. Urinary catechol amine

concentrations as high as this are seldom found even in human cases of pheochromocytoma. In experiments 1—3 (table 8) the adrenaline excretion was higher in rats that had received a smaller water load. As these rats also showed definitely stronger hypoglycaemic signs, it is likely that the increased excretion was not due to renal factors but to a higher sensitivity to insulin. In experiment 4 (table 8), however, where the test animals were injected with a nearly lethal dose of insulin, no such differences could be observed. It may be stated, therefore, that the administration of water in amounts of 2—3 per cent of body weight does not to any significant degree alter the sensitivity to insulin or the excretion of adrenaline in rats. Insulin hypoglycaemia has a diuretic effect, as observed constantly during the course of these experiments. The effect can be noted when the values for the urine flow in figures 20 and 21 are compared. HORRES *et al.* (1950) found adrenaline to be without any effect on the urine flow in rats, although noradrenaline caused a marked increase. Large doses of adrenaline (100 μg.), however, are known to have a diuretic effect in the rat (e.g. GIERE 1954).

The experiment in figure 26 indicated that the adrenaline stores of the body may be totally depleted during life by successive insulin administrations. Similar results have been obtained by HÖKFELT (1951, 1953) and OUTSCHOORN (1951). The low adrenaline excretions elicited by repeated insulin injections (figures 25 and 26) are most readily understood, therefore, when they are considered to be a reflection of the state of the adrenaline stores of the body. The restoration rate of about 6 days for the insulin-induced adrenaline excretion (figure 27) was identical to that found by HÖKFELT (1951, rat) for the restoration of the suprarenal catechol amine stores. UDENFRIEND *et al.* (1953, rabbit) also found that one-half of the adrenaline that disappeared after an administration of insulin was restored in three days. The changes in the adrenaline excretion also in this respect presumably are to be regarded as indicators of the amount of adrenaline present in the suprarenal medulla.

The experiments (figures 25—27) show further that rats with-

out appreciable stores of adrenaline are able successfully to combat insulin in doses that are capable of producing severe hypoglycaemic signs in the intact animals. This is in accord with the results of ZUCKER and BERG (1937 b, cat), VOGT (1951, rat) and VOLK *et al.* (1952, dog) who found that the sensitivity of the test animals to insulin was not altered or was only somewhat increased after adrenal demedullation.

The greatest portion of adrenaline is excreted during the first two hours after the injection of insulin (figures 16 and 29). These results confirm those of YEN *et al.* (1933) and HOLZBAUER and VOGT (1954 a, b), who followed in the dog the changes in the adrenaline content of the blood. The high adrenaline excretion coincided with the decline of the blood sugar level. In some of the experiments the restoration of the blood sugar level occurred without any detectably increased excretion of adrenaline. Apparently adrenaline does not participate in the restoration process. A contrary view has been expressed even lately by, e.g., KEHLER (1954).

It has been an almost uniform finding in the studies in which changes in the adrenaline content of the suprarenals have been followed after an administration of insulin that the adrenaline content continues to decrease in the course of several hours (HÖKFELT 1951, rat, BURN *et al.* 1950, rat, OUTSCHOORN 1952, rat). This occurs also long after the blood sugar level has returned to normal (OUTSCHOORN 1952). In the present studies, however, no increase in the adrenaline excretion was observed over such a long period.

Starvation and the composition of the diet have a profound effect on the carbohydrate metabolism. The subject has been widely discussed by, e.g., CHAMBERS (1938) and PETERS (1945). Animals kept on a diet rich in carbohydrate show a normal tolerance to glucose and a high sensitivity to insulin, whereas those fed a low-carbohydrate diet are highly resistant to insulin and their tolerance to glucose is lowered. This has also been shown to occur in rat (BAINBRIDGE 1925, ROBERTS and SAMUELS 1943). The glycogen content of the liver in the rat is also dependent on the diet. The initial content is higher

in rats on a high sugar diet but it decreases rapidly during the first 24 hours of fast, whereas in the protein-fed rats considerable amounts of glycogen are found in the liver after a fast of 24 hours (MIRSKI *et al.* 1938). The liver glycogen in rats on a mixed diet is depleted after starvation for 24 hours (e.g., NIMS and SUTTON 1954, RUSSELL and BLOOM 1956). During starvation the ability of the organism to burn and store carbohydrates is low, as shown by the decreased tolerance to glucose in the fasting animal (WINTER 1946, rat). Also the resistance to insulin is increased during starvation.

The stronger hypoglycaemic signs, the higher mortality and the more pronounced fall of the blood sugar level after the administration of insulin (tables 10 and 11) show that the rats kept on a carbohydrate-rich diet were also in these studies more sensitive to insulin than the rats on a carbohydrate-poor diet. The higher insulin-induced excretion of adrenaline in the sugar-fed groups may most readily be considered to be a sign of this increased sensitivity (table 10, figure 24). The more pronounced decline in the excretion during the first day of fasting (figure 24) in the rats on a carbohydrate-rich diet as compared to those on a carbohydrate-poor diet (figure 24) and on a mixed diet (figures 22 and 23) reflects a more rapidly developing insulin resistance. The changes in the blood sugar level (table 11) suggest the same.

In these studies the difference in the insulin sensitivity between the sugar-fed and protein/fat-fed rats was more pronounced than in the studies of ROBERTS and SAMUELS (1943). The fasting time of the rats in the studies of ROBERTS and SAMUELS was 34 hours, as compared to 10—20 hours in the present study. The time allowed for the rats to become adapted to the lack of sugar due to the starvation and to acquire an insulin resistance was therefore longer in the studies of ROBERTS and SAMUELS. As shown in figure 24, the decrease in the insulin sensitivity was in the sugar-fed rats particularly marked during the first 24 hours of fast and thus the sensitivities in the two groups approach each other rapidly.

The low insulin-induced adrenaline excretion during the first

few hours of fast was obviously due to intestinal sugar resorption and ensuing diminution of the blood sugar fall (figures 22, 23).

The adrenaline content of the suprarenals of the rat is not altered during a fast of 72 hours (HÖKFELT 1951). The decline in the insulin-induced adrenaline excretion during starvation (table 9, figures 22 and 23) evidently reflects the increase in the insulin resistance due to fasting. The adrenaline excretion in the rats fed a mixed diet (table 9, figures 22 and 23) began to decrease after the first day of fasting. This coincides with the depletion of the glycogen stores of the liver.

When allowed to produce hypoglycaemia, insulin activates the adrenal cortex. This has been shown in man by, e.g., BLISS *et al.* (1954) and DOHAN *et al.* (1955), who found an elevated 17-hydroxycorticoid level in the blood after insulin administration. The insulin resistance in man, which was found by SOMOGYI (1950) to develop during the hypoglycaemia induced by previous insulin administrations, may therefore be explained by a mobilisation of insulin-antagonistic hormones from the suprarenal cortex. In studying the changes in the suprarenal ascorbic acid content and also using histochemical methods, GERSHBERG and LONG (1948) and VOGT (1951) have observed a release of cortical hormones during insulin hypoglycaemia also in rat. In the present studies, however, no indications of a decrease in the insulin activity was observed when insulin was administered repeatedly at two hours' intervals during a hypoglycaemic state. On the contrary, the progressive fall in the blood sugar level, the definite increase in the adrenaline excretion and the poor condition of the test animals suggest a cumulative action of insulin in the rat. The lower minimum level of blood sugar and the larger total excretion of adrenaline suggest that the effect of insulin is greater when administered over a period of six hours than when administered as a single injection (table 12, figures 28, 29 and 30). It has been shown also that an acute administration of cortisone (BURNS *et al.* 1953), 17-hydroxycortisone (PERSKY *et al.* 1955) and adrenocorticotrophic hormone (KESSLER *et al.* 1956) is relatively ineffective in increasing the insulin resistance in man.

Bilateral adrenalectomy in man lowers the excretion of adrenaline to approximately 20 per cent from normal, without any decrease in the noradrenaline excretion (v. EULER, FRANKSSON and HELLSTRÖM 1954). It is evident, therefore, that most of the adrenaline in urine is derived from the suprarenals. Organs such as the spleen, heart and liver (v. EULER 1950, cattle, HÖKFELT 1951, rat) contain adrenaline in amounts that are only a few per cent of the respective noradrenaline content. At the most 1 per cent of the adrenaline in the organism is thus in organs other than the suprarenals. It is, therefore, understandable that no elevation was observed in the present studies during insulin hypoglycaemia in adrenalectomised rats. Evidently the extra-adrenal chromaffin tissue and the sympathetic nervous system are not able to secrete adrenaline into the blood in any appreciable amounts. A small increase in the adrenaline excretion in the present studies, however, may well have been masked by an incomplete emptying of the bladder (table13).

Insulin hypoglycaemia, on the other hand, has been shown to pronouncedly elevate — about 25-fold — the adrenaline content of the liver and heart in the intact rat, which is postulated to be a sign of an increased local synthesis of adrenaline (HÖKFELT 1951). The amounts involved, however, are very small and therefore need not be detectable in urine.

The adrenalectomised rats in the present study were maintained with daily injections of cortisone. This hormone is known to lower the excretion of noradrenaline in man (LUFT and v. EULER 1952). The amounts used in the present study, however, were relatively small, since only the largest of the doses normalised the insulin sensitivity of the test animals.

The mean basal adrenaline and noradrenaline excretion in the adrenalectomised rats maintained on cortisone was somewhat lower than that found in intact rats (table 14). It cannot be stated with certainty whether the difference was due only to a relaxation of the bladder and to the consequent retention of urine, or whether a decrease in the adrenaline and noradrenaline excretion actually occurred in the adrenalectomised animals.[1])

[1]) No increase in the adrenaline excretion was observed in two adrenalectomised patients maintained on cortisone after insulin administration (ELMADJIAN, F., LAMSON, E. T., FREEMAN, H., NERI, R. & VARJABEDIAN, L.: J. Clin. Endocr. 1956, *16*, 876). No data were given, however, concerning the depth of the hypoglycaemia.

DETERMINATION OF ADRENALINE AND NORADRENALINE IN THE URINE IN THE DIAGNOSIS OF PHEOCHROMOCYTOMA

Earlier Investigations

Owing to the largely varying symptomatology the clinical diagnosis of pheochromocytoma has offered great difficulties. Because of this, various pharmacological tests have been used as aids in the diagnosis. The histamine (ROTH and KVALE 1945), benzodioxan (GOLDENBERG *et al.* 1947) and regitine (GRIMSON *et al.* 1949) tests have been most widely used. However, false positive and negative responses are known to occur (GOLDENBERG and ARANOW 1950, PLACE 1951, SACK and KOLL 1954). Endeavours have been made, therefore, to work out more reliable and specific methods where the adrenaline and noradrenaline contents of the blood and urine are determined.

Elevated amounts of pressor substances have been found in blood in cases of pheochromocytoma. BEER *et al.* (1937) tested on a denervated rabbit's ear vein the pressor activity of the blood drawn during a hypertensive crisis from a patient with paroxysmal hypertension. A pronounced vasoconstriction was observed as compared to normal samples. The constriction was reversed by ergotamine. Adrenaline was demonstrated during a hypertensive episode in blood also by HYMAN and MENCHER (1943) on a dog's tail preparation and on cat's denervated eye. SNYDER and VICK (1947) on the other hand, were not able to find adrenaline in the blood by biological means in a verified case of pheochromocytoma.

Also chemical methods have been used in the detection of catechol amines in the peripheral blood in the diagnosis of pheochromocytoma. With the colorimetric method of v. EULER, based on the decolouration of methylene blue under the influence of adrenaline, STRÖMBECK and HEDBERG (1939) found during a hypertensive attack an adrenaline content of more than 1000 times normal. Between attacks, values about 30 times normal were obtained. MORTELL and WHITTLE (1945) made an attempt to estimate the adrenaline content of the blood by the method of RAAB (1943), which is comprised of an aluminium hydroxide adsorption

and reduction of arsenomolybdic acid, but obtained a negative result. VIERSMA *et al.* (1949) found no sympathomimetic activity in the blood in a case of pheochoromocytoma tested on a decerebrated cat but he was able to find more colour-producing substances with the KOBRO modification of the silicic acid-arsenomolybdic acid method of WHITEHORN (1935).

The methods based on the fluorescence reaction have proved more serviceable for the detection of the elevated adrenaline and noradrenaline content of the blood in pheochromocytoma. With the semiquantitative method of v. HUEBER (1940), in which adrenaline is purified by dialysis against a weakly acid solution, VOLHARD (1944) found an adrenaline content of twice normal. ESPERSEN and DAHL-IVERSEN (1946) found a still slightly greater increase by a similar method introduced by KALAJA and SAVOLAINEN (1941). It remained, however, for LUND (1950) to introduce a reliable and sensitive method where both adrenaline and noradrenaline can be estimated accurately in cases of pheochromocytoma. The method has proved to be of great value in the diagnosis of this disease. An elevated adrenaline and mainly noradrenaline content in the blood has been observed with this method in 5 cases during a hypertensive attack (LUND 1952). The level, however, may be normal between attacks.

Also with the method of WEIL—MALHERBE and BONE (1952) a noradrenaline content four times normal has been observed in the peripheral blood in a case of pheochromocytoma (RICHARDSON *et al.* 1955). The method consists of an oxidation of adrenaline and noradrenaline and a subsequent condensation with ethylene diamine whereby fluorescent compounds are formed. Also MANGER *et al.* (1954) found with a modification of this method elevated amounts of catechol amines in the blood in cases of pheochromocytoma but also in patients with azotaemia.

The increased excretion of biologically active catechol amines in urine in cases of pheochromocytoma was first reported by ENGEL and v. EULER (1950). The diagnosis, first of two cases (ENGEL and v. EULER 1950) and later of four cases (v. EULER 1951) with this means, has widely stimulated the research in this field. The method of v. EULER and HELLNER (1951) has been found reliable and its diagnostic significance is already well established (HEDLY and LUBLIN 1951, HAMILTON *et al.* 1953, BURN 1953, de GRAEFF *et al.* 1953, PEART 1954, ISRAELSKI *et al.* 1954, DAVIS *et al.* 1955, HELPS *et al.* 1955, RICHARDSON *et al.* 1955). In cases of a secreting tumour of the chromaffin tissue enormous amounts of adrenaline and noradrenaline are excreted frequently. In addition to these specific biological methods, less tedious but also less accurate methods have been used, consisting of injections of untreated urine samples into test animals, with blood pressure recordings (CARL *et al.* 1951, cat, v. EULER 1952, cat, WEIMAN *et al.* 1954, dog). MOULTON and

WILLOUGHBY (1955) were able by a similar procedure to screen 6 out of 7 cases of pheochromocytoma.

Owing to greater difficulties in the urinary catechol amine determinations the results with the colorimetric methods are not as reliable. BEYER *et al.* (1951), using the colorimetric iodoadrenochrome method of BEYER and SHAPIRO (1945), could not find elevated amounts of catechol amines in urine in two cases of pheochromocytoma, while in a third case after a hypertensive crisis large amounts of colour-producing substances were found that could be determined quantitatively.

The increased urinary excretion of adrenaline and noradrenaline can more accurately be detected chemically by the methods based on the fluorescence reaction. LUND (1952) was able to show in 4 cases of pheochromocytoma a highly elevated urinary adrenaline and noradrenaline concentration. The daily excretion in these cases varied between 420—3000 μg. noradrenaline and 105—750 μg. adrenaline. The non-specific fluorescent substances in most samples do not interfere with the estimation at these highly increased adrenaline and noradrenaline levels, as shown by GOLDENBERG *et al.* (1954) in studies that comprised 16 cases of pheochromocytoma. In one case, however, a falsely negative result was observed when the method of LUND was used (GOLDENBERG *et al.* 1954). With the methods based on the chromatographic purification of the urinary catechol amines (HAMBERG and v. EULER 1950, GOLDENBERG *et al.* 1954, GOLDSTEIN and ABELIN 1956) these substances can be accurately determined by both biological and fluorimetric means.

Own Investigations

In the course of the studies described in the earlier sections a total of 356 clinical samples were analysed for the main purpose of finding cases of pheochromocytoma. The determinations were carried out by means of the fluorescence reaction. Various adjustments were made in the procedure, as described in earlier sections, and applied to the estimations as soon as they were found suitable. The values for the adrenaline and noradrenaline excretion obtained in this material were therefore obtained with several modifications of the method and are not comparable with each other.

Most of the samples were collected during 24 hours and brought immediately for analysis. To protect adrenaline and noradrenaline from destruction in the case of lengthy transportation, the urine samples were acidified to pH 4.

A total of 154 urinary specimens were analysed according to the modification with butyl alcohol extraction, high dilution of the eluate, and differentiation between adrenaline and noradrenaline, as described on pages 37 and 38. The mean adrenaline and noradrenaline excretion in this group was 33 μg. in 24 hours, with an adrenaline percentage of 29 per cent. After hydrolysis a mean excretion of 68 μg. in 24 hours was obtained. In seven specimens the fluorescence reaction was strong enough to correspond to an excretion of more than 100 μg. adrenaline and noradrenaline in 24 hours in unhydrolysed specimens. In two of these cases a tumour of the chromaffin tissue was found.

Case 1. — A 6-year-old girl had lost weight for six months before admission to hospital and had attacks of sweating, headache, pain in the upper left segment of the abdomen, nausea and vomiting. The blood pressure varied between 130/80—210/150 mm Hg. The regitine test (2.5 mg. intravenously) was negative. An excretion of 920, 1400, 1200, and 1300 μg. noradrenaline in 24 hours was found in the four unhydrolysed samples analysed. In the differentiation test no adrenaline could be detected in any of the samples. The tumour was multiple, as established by autopsy.

Case 2. — A pheochromocytoma had been removed from an adult female six years previously. She was hospitalised for examination because of recurrence of the symptoms. Two urine samples that were collected during a normotensive period had a normal adrenaline and noradrenaline content (30 and 50 μg. in 24 hours). For diagnostic purposes a hypertensive attack was induced by abdominal massage. The urine collected during this attack contained adrenaline and noradrenaline in definitely elevated amounts (13 μg. per 100 cc., after hydrolysis 21 μg. per 100 cc.). The proportion of adrenaline was 5—10 per cent. An extra-adrenal pheochromocytoma was found at operation.[1])

In three of the remaining five cases with an elevated adrenaline and noradrenaline excretion the subsequent samples showed a normal adrenaline and noradrenaline content. Neither did the clinical observations support a diagnosis of pheochromocytoma. One of these three cases was later operated on and

[1]) I am grateful to PEHR MALM, M.D., and JARL FORSSELL, M.D., for the clinical information on this case. It will be published by them in detail elsewhere.

renal atrophy only was found. The possibility of the existence of a secreting tumour in the other two cases has not yet been definitely excluded.

In the 147 cases that showed a normal catechol amine excretion in one or several urine samples, inquiries were made concerning the clinical history of 111 patients. The material mostly comprised different forms of hypertension, neuro-circulatory dystonia and hyperthyroidism. No cases of pheochromocytoma were found. In this group three autopsies were performed without the finding of a tumour. The clinical picture in seven of the cases so closely suggested the presence of a secreting chromaffin tumour that an intra-abdominal operation was performed, with a negative result.

A total of 201 analyses were carried out by the various earlier modifications of the method. In this group the mean adrenaline and noradrenaline excretion was 81 μg., and in hydrolysed specimens 151 μg. in 24 hours. In 17 patients values occasionally over 200 μg. in 24 hours were obtained, and in three of these a pheochromocytoma was found.

Case 3. — This case of a 6-year-old boy has been published elsewhere (SULAMAA and WALLGREN 1955). The catechol amine excretion on different days was 500, 610, 920, 1750 (hydrol. 2250), and 1250 (1200) μg. in 24 hours. The fluorescence was due to noradrenaline. After the operation the clinical signs vanished and the catechol amine excretion has since been at the normal level.

Case 4. — A 10-year-old boy had lost weight during half a year, with recurrent attacks of headache and vomiting. The blood pressure was stable at 210/160 mm Hg. The benzodioxane test was negative. In the regitine test the blood pressure fell from 225/200 to 200/165 mm Hg. The catechol amine excretion on different days was 110, 140, 130 and 240 μg. in 24 hours. In these samples, however, a particularly strong inhibition of the fluorescence reaction was encountered. As in such samples the proportion of non-specific fluorescence is relatively increased, the excretion was considered to be only sligthly increased. At operation the right adrenal was found to be twice the normal size. It was removed and sympathectomy and splanchnic nerve resection were performed. Histological examination of the adrenal revealed in the medulla hyperplastic islets of chromaffin cells. The clinical state of the patient improved postoperatively. The blood pressure, however, began to decline only after a few months. Postoperatively the adrenaline and noradrenaline excretion was

20 and 50 μg. in 24 hours. However, these cannot be compared with the preoperative values, as they were determined by another modification of the method.

Case 5. A further case has already been published (PEKKARINEN and PITKÄNEN 1955 b). This was a 24-year-old female, in whom the excretion varied between 430—2400 μg. in 24 hours (in hydrolysed samples 470—1750 μg. in 24 hours) and was composed totally of noradrenaline. A paraganglioma was found close to the abdominal aorta.

Inquiries were made concerning the clinical history of 129 cases in this group. No additional cases of pheochromocytoma were found. In five cases an intra-abdominal operation or an autopsy was carried out with a negative result.

Conclusions

The present series studied by means of the fluorescence reaction included five verified cases of pheochromocytoma. In three of these cases (cases 1, 3 and 5) the urinary adrenaline and noradrenaline excretion was almost constantly on the very high level of about 1000 μg. in 24 hours. The nonspecific substances known to interfere in the methods used in the present work do not disturb at this catechol amine concentration. In most of the cases in the literature in which the urinary adrenaline and noradrenaline excretion has been estimated it has been shown to exceed 500 μg. in 24 hours (v. EULER 1951 six cases, HEDLY and LUBLIN 1951 one case, LUND 1952 four cases, BURN 1953 one out of three cases, deGRAEFF *et al.* 1953 one case, HAMILTON *et al.* 1953 four out of five cases, GOLDENBERG *et al.* 1954 thirteen out of sixteen cases, ISRAELSKI *et al.* 1954 one case, DAVIS *et al.* 1955 one case, HELPS *et al.* 1955 three cases, JORDE 1956 one case). An excretion that is so greatly elevated is, especially if continuous, considered to be pathognomonic for this disease and the diagnosis can well be established before operation solely on this basis.

In one of the cases (case 4) the adrenaline and noradrenaline excretion was only occasionally elevated well over the normal level. As the determinations were performed with a modification that has to be considered rather unspecific, the real increase in

the adrenaline and noradrenaline excretion was not so clearly visible. The actual increase in the excretion in this case was, at any rate, considerably less than in the first mentioned three cases.

Case 2 is the most interesting from the point of view of diagnosis. In the light of this case it seems likely that a normal adrenaline and noradrenaline excretion does not definitely exclude the possibility that a pheochromocytoma with secretory capacity is present. To diagnose these cases where there is only occasional secretion from the tumour, the provocation of an attack with histamine or massage, combined with determination of the excretion of adrenaline and noradrenaline during the attack, seems well indicated.

In the series of cases in which a normal adrenaline and noradrenaline excretion was found, the clinical examination also revealed no additional cases of pheochromocytoma. Since, however, the differential diagnosis of the disease often is impossible and since obviously in some cases a normal adrenaline and noradrenaline excretion can occasionally be observed, it cannot be regarded as proved that the series did not include additional cases of pheochromocytoma. The few intra-abdominal operations performed suggest, however, that their number could not have been great.

The mean adrenaline and noradrenaline excretion of 81 μg. in 24 hours obtained by the earlier modifications of the method was remarkably higher than that found by v. EULER and HELLNER (1951) by the specific biological method (30 μg. in 24 hours). The lower mean adrenaline and noradrenaline excretion (33 μg. in 24 hours) obtained with the procedure where the butyl alcohol extraction, the high dilution of the eluate and the more specific blanks were used indicates the importance of these steps in the specificity of the method. Less pronounced increases in the adrenaline and noradrenaline excretion can thus be detected. On the other hand, only in a few samples an occasional unspecific increase in the excretion was observable. The fluorescence method, therefore, is to be considered serviceable in the differential diagnosis of pheochromocytoma.

STATISTICAL TREATMENT OF CERTAIN DATA

In determining *normal range of variation* on the basis of control material, the mean

$$(1) \quad \bar{x} = \Sigma \, x/n$$

and the standard deviation

$$(2) \quad s = \sqrt{\frac{\Sigma \, x^2 - (\Sigma \, x)^{2}/n}{n-1}}$$

have been computed. In the formulas x stands for the variate under investigation, n for the number of observations. To obtain the width of e.g. 95 per cent normal range, the standard deviation is to be multiplied by the so-called t-variable corresponding to the number of observations and to the desired confidence level 95 per cent. Result of this multiplication subtracted from and added to the mean gives the limits of normal variation in such a way that approximately 5 per cent of normal observations will lie outside these limits. The t-values to be applied have been obtained from appropriate statistical tables (FISHER and YATES 1953). Results:

	Human material		Animals	
	Total catechols	Adrenaline percentage	μg.	%
Number of observations	18	18	33	33
Mean	54	33	0.36	47
Standard deviation	15	22	0.16	14
Lower 95 per cent limit	23	—	0.03	18
Upper ——„——	86	80	0.68	77

Effect of hydration has been analysed by means of statistical variance analysis. This type of analysis (see e.g. FISHER 1950) enables the investigator to form a combined estimate of random variation present, so as to evaluate the significance of observed differences between experimental groups. Further, variation between replicated experiments or parallel experiments can be eliminated so that it does not affect the analysis in question. Concerning the effect of hydration, three experiments have been performed; within each experiment there are two hydration groups (< 3 cc. and > 6 cc.). Variation between experiments has been eliminated, whereafter differences between hydration groups have been statistically tested both separately in each expriment and as a pooled difference. Calculations of analysis are exposed in Table 15. The analysis results in that no statistically significant difference can be established between hydration groups.

Table 15. Effect of hydration: analysis of variance.

Source of variation	Sum of squares	Degrees of freedom	Variance	Variance ratio	P
Differences between experiments	9.6204	2	4.8102	36.92	0.0005
Differences between hydration groups:					
Exp. I	0.1773	1	0.1773	1.36	...
Exp. II	0.1654	1	0.1654	1.27	...
Exp. III	0.3599	1	0.3599	2.76	...
Total	0.7026	3	0.2342	1.80	...
Random variation ..	2.2143	17	0.1303	...	...
	12.5373	22	...	...	...

For column heads and entities in the analysis table statistical textbook can be consulted (e.g. FISHER 1950). Variance ratio shown in the table is used as a test variable: if it is "too large", the difference in question can be considered statistically significant. The figure P indicates the probability that the observed difference could be a result of chance only. If P is small, e.g. less than 0.05, statistical significance is said to be established. P's larger than 0.05 have been omitted in the table, for their numerical values are not of particular interest.

Effect of starvation has been analysed essentially in the same way as described in the foregoing. Generally, the result supports strong evidence in favour of existence of a real effect of starvation upon adrenaline excretion. The analysis is shown in Table 16.

Table 16. Effect of starvation: analysis of variance.

Source of variation	Sum of squares	Degrees of freedom	Variance	Variance ratio	P
Differences between experiments	27.5279	5	5.5056	43.87	0.0005
Differences between starvation groups:					
Exp. 1	2.5599	1	2.5599	20.40	0.0005
Exp. 2	1.2099	1	1.2099	9.64	0.005
Exp. 3	—	1	—	...	...
Exp. 4	0.0252	1	0.0252	/4.98/	...
Exp. 5	1.5766	1	1.5766	12.56	0.005
Exp. 6	0.1562	1	0.1562	1.24	...
Total	5.5238	6	0.9206	7.34	0.0005
Random variation ..	3.2640	26	0.1255	...	...
	36.3157	37	...	...	

Effect of diet also has been analysed as in the foregoing. The analysis reveals high statistical significance, mainly due to Experiment 3:

Table 17. Effect of diet: analysis of variance.

Source of variation	Sum of squares	Degrees of freedom	Variance	Variance ratio	P
Differences between experiments	7.7607	2	3.8804	10.22	0.001
Differences between diet groups:					
Exp. 1	0.1433	1	0.1433	/2.65/	...
Exp. 2	1.2216	1	1.2216	3.22	...
Exp. 3	14.5111	1	14.5111	38.22	0.0005
Total	15.8760	3	5.2920	13.94	0.0005
Random variation ..	7.9739	21	0.3797	...	...
	31.6106	26	...	...	...

Comparison of two groups for which different insulin administration pattern was applied, has been performed by means of *t*-test. If in each group the sum of squares is denoted by

(3) $$Q = \Sigma x^2 - (\Sigma x)^2/n,$$

a pooled estimate of the standard error of the difference of the two means is obtained by

(4) $$\varepsilon_{\triangle} = \sqrt{\frac{n_1 + n_2}{n_1 n_2} \cdot \frac{q_1 + q_2}{n_1 + n_2 - 2}},$$

where the sub-indices 1 and 2 refer to the two groups each. Now the test is performed by dividing the difference of means into the standard error of the difference, thus:

(5) $$t = (\bar{x}_1 - \bar{x}_2) / \varepsilon_{\triangle}.$$

Significance of the observed *t*-value can then be determined with the aid of appropriate statistical tables as mentioned already, remembering that there are $n_1 + n_2 - 2$ degrees of freedom. Results:

Means to be compared	1.97	271
Difference of the means	0.74	
Standard error of the difference	0.26	
Test variable *t*	2.86	
P	0.05	

GENERAL SUMMARY

The object of the present investigation was to work out a specific chemical method for the determination of adrenaline and noradrenaline in human urine and to apply the method to experimental and clinical use. The method consisted of an adsorption of adrenaline and noradrenaline onto aluminium oxide, followed by elution with sulfuric acid. Following the oxidation of the eluates, the fluorescence reaction of adrenaline and noradrenaline was developed by the addition of alkali and stabilised with ascorbic acid. A high dilution and butyl alcohol extraction of the eluate and the use of more specific blanks were suggested for the elimination of the disadvantages that were found to handicap the method.

The adrenaline and noradrenaline excretion values obtained by the fluorescence method correlated well with those obtained by biological assay but were constantly somewhat higher. Because of the high dilution of the eluate and the presence of non-specific fluorescence, the method has only a limited value in the analysis of urine specimens with a low adrenaline and noradrenaline content.

The fluorescence reaction was found suitable for the quantitative estimation of the elevated adrenaline and noradrenaline content also in the urine of the rat. Experiments were made in which the changes in the urinary adrenaline and noradrenaline excretion were followed during insulin-induced hypoglycaemia in the rat under various experimental conditions. In these experiments changes in the blood sugar level and in the suprarenal catechol amine content were also observed.

Insulin hypoglycaemia resulted in a 10—20-fold or greater increase in the excretion of adrenaline from the basal adrenaline

and noradrenaline excretion level, with no detectable increase in the noradrenaline excretion. Most of the adrenaline was excreted during two hours following a subcutaneous injection of insulin. The adrenaline excretion was independent of the rate of urine flow.

The proportion between the insulin dosage and the excreted adrenaline was stoichiometric over a wide range.

The suprarenal catechol amine stores were depleted by repeated insulin administrations. This was reflected in a marked diminution in the insulin-induced adrenaline excretion. The excretion increased again simultaneously with the restoration of the adrenaline stores.

The insulin-induced adrenaline excretion was lower in rats kept on a diet rich in protein and fat than in the group fed previously a carbohydrate-rich diet. During starvation the excretion declined, particularly rapidly in rats previously fed a carbohydrate-rich diet. In this connection the adrenaline excretion was regarded as an indicator of the severity of the stress exerted by insulin on the organism.

Insulin was shown to have a cumulative action in the rat when administered subcutaneously at two-hour intervals.

The adrenaline excretion was not increased during insulin hypoglycaemia in adrenalectomised rats.

The clinical series analysed comprised five cases of pheochromocytoma. In three of these cases the total urinary adrenaline and noradrenaline excretion was about 1000 μg. in 24 hours. In the fourth case the excretion was occasionally 200—300 μg. in 24 hours, at the most. In the fifth case an elevated noradrenaline excretion occurred only during a hypertensive attack induced by abdominal pressure.

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